Development of Mass Spectrometric Methods for the Identification and Quantification of Allergenic Milk and Hazelnut Proteins in Food

by

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Abstract

Immunoassays are the method of choice for fast detection of food allergens. The first step in their development is the production of antibodies (ABs). The characterisation of these ABs due to their properties has to be carried out well. Therefore, suited hazelnut protein preparations were produced in this study for characterisation of in-house produced monoclonal (mABs) and polyclonal (pABs) antibodies. For this purpose, various extraction and isolation strategies were used. These extracts were purified with different chromatographic methods; finally the best results can be achieved with a two-step FPLC method (affinity and reversed phase chromatography). Gel electrophoresis was used for the first controlling of protein profiles before they were further characterised by western blot. The mABs showed identical protein recognition patterns, which were analogue to the pattern of pAB from mouse. Also pABs from rabbit and chicken showed similar recognition pattern.

Although immunoanalytical methods are rapid and sensitive, they have some drawbacks. Therefore, the emphasis of this study was put on developing MS-based methods, complementary to common immunoassays, for the detection of milk and hazelnut allergens in food samples. In this context, the determination and selection of peptides, which could function as markers for allergen-identification, played an important role. For this purpose, different protein databases and bioinformatic tools had to be used to get information about expected peptides and to identify the specificity. The analytical data obtained by combination of reversed-phase high performance liquid chromatography and quadrupole tandem mass spectrometry led to identification of several tryptic peptides in milk and hazelnut samples, which can be used as marker. Among them, seven peptides from four milk allergens and eight from three hazelnut allergens, with the highest MS-signal were synthesized and used as standards for calibration curves to develop multi-analyte LC-MS/MS methods in selected reaction monitoring (SRM) mode for the detection and in part quantification of milk or hazelnut allergens in food samples. With these methods, LODs down to 1.1 ng/mL could be achieved and in spiked samples, the marker peptides could be detected with high recoveries.

Abstrakt

Immunoassays sind die Methode der Wahl für die schnelle Detektion von Lebensmittelallergenen. Der erste Schritt in ihrer Entwicklung ist die Produktion von Antikörpern (AK). In dieser Arbeit wurden geeignete Haselnuss Präparate für die weitere notwendige Charakterisierung von in-House produzierten Antikörpern hergestellt. Zu diesem Zweck wurden unterschiedliche Extraktions- und Isolierungsstrategien verwendet. Die Extrakte wurden mit verschiedenen chromatographischen Methoden gereinigt; schließlich wurden die besten Ergebnisse mit einem Zwei-Schritt-Chromatographie-Methode (Affinitäts- und Reversed Phase Chromatographie) erreicht. Gel-Elektrophorese und Western-Blot wurden für die Kontrolle der Protein-Profile und deren Charakterisierung eingesetzt. Die monoklonalen Antikörper zeigten identische Protein-Erkennungsmuster, analog zu dem Muster des Mausserums. Auch polyklonale Antikörper aus Hase und Huhn zeigten ähnliche Erkennungsmuster.

An sich sind immunoanalytische Methoden schnell und empfindlich, aber aufgrund mancher Einschränkungen wurde der Schwerpunkt dieser Arbeit auf der Entwicklung von MS-basierten Methoden für den Nachweis von Milch- und Haselnuss- Allergene in Lebensmitteln gelegt. In diesem Zusammenhang spielte die Bestimmung und Auswahl der Peptide, die als Marker für die Allergen-Identifizierung verwendet werden konnten, eine wichtige Rolle. Verschiedene Protein-Datenbanken und Bioinformatik-Werkzeuge wurden benützt, um Informationen über zu erwartende Peptide zu erhalten und um die Spezifität zu identifizieren. Die erhaltenen analytischen Daten von LC-MS/MS führten zur Identifikation von mehreren Peptiden in Milch und Haselnuss, die als Marker verwendet werden können. Unter ihnen wurde sieben Peptide aus vier Milch Allergenen und acht Peptide aus drei Haselnuss Allergenen mit jeweils höchsten MS-Signalen synthetisiert und als Standards für die Kalibrierkurven benutzt. Das führte in weiterer Folge zur Entwicklung von Multianalyt LC-MS/MS-Methoden im SRM-Modus für die Detektion und Quantifizierung von Milch oder Haselnuss Allergenen in Lebensmitteln. Mit diesen Methoden konnten LODs bis zu 1,1 ng/mL erreicht werden. In gespiked Proben konnten die Markerpeptide mit hohen Wiederfindungen erkannt werden.

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List of abbreviations

AB,ABs,mAB,pAB	Antibody, antibodies, monoclonal antibody, polyclonal antibody
ALA	α-lactalbumin
BLG	β-lactoglobulin
CE	Capillary electrophoresis
CAD	Collisionally activated dissociation
CID	Collision induced dissociation
dc	Direct current
DNA	Desoxyribonucleic acid
EAACI	European Academy of Allergology and Clinical Immunology
EFSA	European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assay
ESI	Electrospray ionisation
EU-VITAL	European Voluntary Incidental Trace Allergen Labelling
FPLC	Fast protein liquid chromatography
GC	Gas chromatography
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
IEC	Ion exchange chromatography
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IT	Ion trap
LC	Liquid chromatography
LOD	Limit of detection
m/z	Mass to charge
MALDI	Matrix-assisted laser desorption
MS, MS/MS	Mass spectrometry, tandem mass spectrometry
MW	Molecular weight
nsLTPs	Non-specific lipid transfer proteins
OPD	*o*-phenylenediamine
PCR	Polymerase chain reaction
Q, QqQ	Quadrupole mass analyzer, triple quadrupole instruments
rf	Radio frequency
RPC	Reversed phase chromatography
SDS-PAGE	Sodium dodecylsulfate - polyacrylamide gelelectrophoresis
SEC	Size exclusion chromatography
SRM	Selected reaction monitoring
TMB	3,3´,5,5´-tetramethylbenzidine
TOF	Time-of-flight

1 Objective of this work

This work was part of the Christian Doppler Laboratory for Rapid Test Systems for Allergenic Contaminants, which is engaged in a wide range of scientific studies of allergenic food proteins from antibody production to developing different analytical tools (both rapid immunoanalytical methods such as ELISA and lateral flow devices, and analytical methods based on MS) for the identification and characterisation of allergens in foods. A major focus in this laboratory is put on allergens, which are listed in Annex IIIa of the EC-Directive 2007/86/EC and have to be declared on the label of food products. Immunobased assays are the method of choice for fast detection of food allergens; therefore they were discussed in a review paper [1] and commercial available test kits were introduced.

The first and essential step in development of immunoassays is the production of antibodies (ABs), which can specifically recognize defined protein, and their characterisation has to be carried out well. For this purpose, the competitive ELISA was used in our laboratory. In ELISA the blocking is an important step because it can reduce the non specific binding and increase the sensitivity of the ELISA. In case of allergenic proteins, it is better not to use the protein blocking solutions. Therefore, alternative carbohydrates or synthetic blockers were comprised to find out the best combination [2].

A main focus of this work was extraction and purification of allergenic food proteins and their characterisation with sodium dodecylsulfate-polyacrylamide gelelectrophoresis (SDS-PAGE) and mass spectrometry according to their molecular masses. Furthermore these preparations were used for the characterisation of in-house produced antibodies.

Liquid chromatography coupled with electrospray ionisation to tandem mass spectrometry is currently the routine laboratory method for analysis of peptides and proteins. It combines the key benefits of separation of the biological molecules by HPLC and sensitive detection of the analytes by MS. Therefore this technique together with database searches and different bioinformatic tools was used for selection of marker-peptides and developing the MS-based methods for the detection of milk [3] and hazelnut [4] allergens by LC-ESI-MS/MS in SRM mode.

In this thesis after a short definition of allergy, different food allergens are categorized according to their origin, followed by legalisation requirements. Among the different methods, which can be used for the detection of food allergens, the immunoassay ELISA and MS- based technique, which were used in this work, are discussed. The results, which cover both immunobased and analytical-based research fields of food allergens, are presented as four published papers and a paper, which will be submitted; and finally the results are discussed.

2 Introduction

2.1 Allergy

Humans are generally exposed to different foreign substances like chemicals, pollen, house dust, and food components, which are normally harmless, but they can elicit in some sensible individuals hypersensitivity known as allergy [5]. This hypersensitivity is mediated by the interaction of two components: allergens (antigens, which elicit allergic reactions), and antibodies (immunoglobulin) [6] [7]; and consists of two stages, sensitization and elicitation. By the initial contact with the protein, internalization of the foreign protein and production of the antibody takes place. Upon re-exposure, due to recognition of allergen by allergen-specific antibody [8] [9], a variety of mediators such as histamines are released that lead to inflammatory reactions and tissue damages known as allergic reactions [10]. They include a variety of clinical symptoms appeared in skin, respiratory- and gastrointestinal tract for example urticaria (hives), rhinoconjunctivitis, angio-oedema, hypotension, pruritus, atopic dermatitis, colic, vomiting, diarrhoea, asthmatic wheeze [6] [11], and in severe cases the anaphylactic shock [10].

Gell et al. [12] classified the allergic reactions in four groups (Table 1). Characteristic for type I is a rapid or acute response due to the recognition of allergens by immunoglobulin E (IgE), which leads to elevated levels of this antibody [13]. Type I is the only IgE mediated allergy; three other types are non-IgE mediated. Type II and III are mediated by IgG or IgM. Type IV is mediated by CD4 helper T-cells; therefore not an antibody mediated hypersensitivity. Since the clinical symptoms of type IV occurs more than 8 hour after exposure to allergen, it is also known as delayed-hypersensitivity [8]. Although there is symptomatic treatment for allergy e.g. antihistamine tablets [14], but there is no cure for it, and only the strict avoidance of allergens by sensible individuals can prevent the allergic reactions [11].

2.1.1 Food allergy and intolerance

Food allergy is caused by an abnormal response of the immune system to the particular dietary food proteins, which can elicit diverse clinical symptoms (ranges from

mild symptoms to more severe and life-threatening reactions). The immune system-mediated food allergy should be distinguished from other types of food sensitivity.

Table 1: Gell and Coombs [12] classification of allergic reactions types

Type	**Alternative name**	**Mediator**	**Appearance in**	**Clinical symptoms**
I	Immediate hypersensitivity	IgE	< 30 min	• Urticaria (hives) • Conjunctivitis • Allergic rhinitis and asthma • Gastroenteritis • Anaphylactic shock
II	Antibody-dependent cytotoxic hypersensitivity	IgG/IgM	Minutes to several hours	• Autoimmune reactions • Hemolytic anemia • Thrombocytopenia • Goodpasture's syndrome
III	Immune complex hypersensitivity	IgG	3-10 hours	• Alveolitis • Arthus reaction • Systemic lupus erythematosus • Serum sickness • Vasculitis
IV	Delayed-type hypersensitivity	T-cells	8-48 hours	• Contact dermatitis • Chronic transplant rejection • Multiple sclerosis • Atopic dermatitis • Allergic rhinitis and asthma

The European Academy of Allergology and Clinical Immunology (EAACI) classified in a Position Paper [15] the adverse reactions to food according to their mechanisms (Fig 1). These reactions are divided into toxic ones, which can occur in any individual who is exposed to a high enough dose of toxin (e.g. staphylococcal enterotoxins), and non-toxic reactions. The non-toxic reactions are categorized again to immune mediated, to which food allergy belongs, and non-immune mediated (better known as food intolerance) [8]. The latter could be elicit via either enzyme deficiencies (for example lactose intolerance due to lactase deficiency) or pharmacological reactions (e.g. vasoactive amines; they are amino groups containing substances such as histamine

or serotonin that, via their action on the blood vessels, can alter their permeability or can cause vasodilation). The food allergy can be further classified into IgE- and non-IgE-mediated. Since the most important food allergies belong to immediate IgE-mediated hypersensitivity, it is the best characterised form. The delayed cell-mediated immune reactions, to which celiac disease belongs, are less investigated [13].

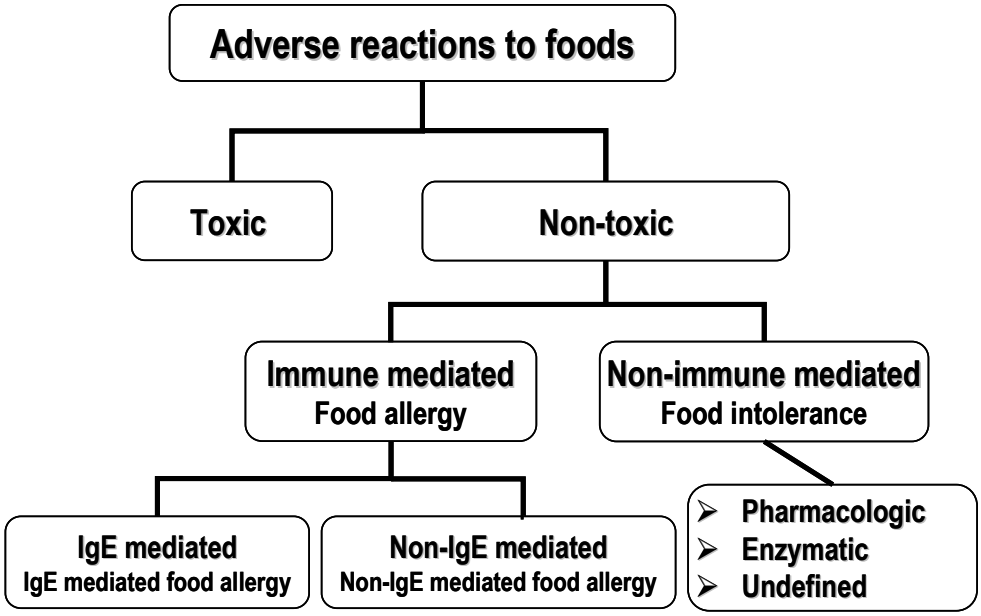

Fig 1: Classification of adverse reactions to food according to EAACI [15]

It is difficult to estimate the accurate prevalence of food allergy overall in the world because it depends on geographical region and food culture/culinary habits of different folks or nations [7] for example allergy to milk and peanut is the prevalent food allergy in USA and fish allergy is frequently observed in Scandinavian countries, Spain and Japan [5]. In the last decade, the prevalence has risen and also the severity of allergy seems to be increasing, especially in industrial countries [8]. The percentage of adults suffering from allergy varies depending on the food (1-4%); the children are even more susceptible with 4-8% [13]. Moreover, some studies showed that the children in rural regions, where exposure to different antigens is higher and therefore the immune system is more challenged, developed less allergies comparing with the children of urban regions. But these results claiming that rural environment is a protection against allergy, could not be confirmed [5].

Although the perceived prevalence of allergy could be as high as 25% of the population, this could not be confirmed after clinical tests [16]. Nevertheless food allergy is considered as a major health problem and due to its incurability, it is essential

for individuals suffering from allergy to avoid the offending food to protect themselves from potentially life-threatening symptoms; something that is not always easy according to the fact that some food stuff could be found in many products [17]. This highlights the need for consumers to get information about the ingredients of food products and their demand for more rigorous food safety testing [11].

2.2 Food allergens

In the field of food allergy, it was reported that sometimes trace amounts of offending food can cause adverse reactions in susceptible individuals and the threshold levels are different from patient to patient [3]. The food allergens are classified into major and minor allergens. If 50% of specific IgE bind the allergen, it defines as major allergen; ≤ 10% refers minor allergens. The major food allergens are seldom eaten raw; they undergo normally different kind of processing such as grinding, drying, heating, chilling, fermentation, hydrolysis, and purification, before they are bought by consumers (pasteurization or sterilization of milk and roasting of nuts are two special examples), or they are cooked before eating [11]. All these processes can alter the structure of allergens and so change the allergenicity. It can result in decreasing the allergenicity by destruction of proteins and changing of the 3-D structure or inactivation of epitopes due to cleavage of the allergens, or respectively increasing the allergenicity as a result of revealing hidden epitopes and therefore a better accessibility or new epitope-formation due to protein-protein interactions [18]. It has to be consider, that certain proteins are more resistant to chemical or thermal treatment and denaturating conditions such as pH changing or high pressure; for example, the prolamin superfamily, whose proteins capable to build disulfide bonds because of presence of six or eight cysteine residue. Some other proteins have the ability to refold themselves again after these treatments and so retrieve their allergenicity [8].

2.2.1 Characterisation of food allergens

Generally, any molecules that enables to elicit the production of antibodies and can react with them is an allergen; however they are mainly from biological resource and the majority of them are proteins [7] [19], which shared some functional and physicochemical properties. It was discussed if these molecular characteristic modifications play a role for their allergenicity. For example, the allergens show often

the ability to bind disulfide bonds and also different types of ligands, to aggregate or form oligomer, and they are usually glycoproteins. Precisely considered, all of these named properties may lead to stability of allergens; it means they can maintain their natural 3-D structure or they can refold after thermal or proteolytic treatment [20]. These normally heat- and digestion resistant allergens can elicit symptoms in the gastrointestinal tract and also sever anaphylactic reactions, however those allergens, which are labile to gastric digestions and heat treatment, trigger symptoms that are often restricted to oral allergy syndrome and mild local symptoms in skin and respiratory tract [21] [22]. Another common property of allergens is their ability to interact with cell membranes or some lipid structures that result in plant protection against pathogens [20].

The allergens can vary in the molecular weight between 3-160 kDa [5], but the antibodies can recognise only a specific part of them, which is known as epitope [23]. Theoretically, any protein could act as allergen and sensitize the immune system; however, 90% of most severe IgE mediated food allergic reactions are elicited by eight main commodities: cereals containing gluten, crustaceans, hen's egg, fish, peanuts, soybeans, cow's milk, and tree nuts [7] [13]. In early childhood, egg white and cow's milk are the major allergens [8], but in most of cases the children can outgrow these allergies [19] and can tolerate them at the age of three [24]. Many of these major allergens are well investigated [7]; the amino acid sequences are identified, the proteins were characterised and even 3D structures are known in some cases. This information is collected in several allergen databases such as Allergome [25] and InformAll [26]. In other protein databases like UniProt [27] valuable data can be found about allergens and their sequences and functions [21].

According to sequence identity (30% and more) or to structure homology and similar functions, the proteins were divided into different families, or into superfamilies if lower sequence similarity but common evolutionary origin appears [14]. Pfam 25.0, a database that collected and classified proteins to different families, showed 12273 protein families in March 2011 [28]. However, comparing the data obtained from different allergen databases proved that the allergens are restricted to certain families and not randomly distributed among the various protein families [8]. Additionally 29 protein families include more than one food allergen [29]. It seems that this restriction is valid also for the other allergens, for example pollen allergens belong only to 29 protein

families [8]. This structure homology can apparently be a reason or at least play a role for cross-reactivity between different allergens; more than 50% sequence identity can be decisive for cross-reactivity [22].

The allergen terminology was regulated according to the accepted taxonomic name of their source: the genus is represented by its three first letters, followed by a space, and then the species is shown with its first letter, again a space, and finally a sequential number of entered new allergen [30]. Writing in italics is used for indication of allergen encoding genes [31]. The food allergen can be classified into allergens from plant or animal sources (Table 2). For example, relative allergen library could include hazelnut, peanut, celery, apple, and peach from plants and cow's- and goat's milk, hen's egg, codfish, carp, and shrimp from animal origin [22].

Table 2: Classification of food allergens according to their origin [14] [22]

Origin	**Major family**	**Subfamilies**	**Function**	**Some examples**
Animal	Tropomyosin		Regulator protein	Pen i 1, Hom a 1
	Parvalbumin		Ca^{2+} -binding	Gad c 1, Sal s 1
	Caseins		Ca^{2+} -binding	Bos d 8
Plant	Prolamin superfamily	Prolamin	Storage protein	Tri a 19, Sec c 20
		2S albumin	Storage protein	Sin a 1, Ber e 1
		nsLTP	Plant defence system	Pru p 3, Mal d 3
		α-amylase/ protease inhibitor	Plant defence system	Hor v 15, Sec c 1
	Cupin superfamily	7S globulin	Storage protein	Ara h 1, Jug r 2
		11S globulin	Storage protein	Ara h 3, Cor a 9
	Bet v 1		Storage protein	Mal d 1, Pru av 1
	Profiling		Regulator protein	Mal d 4, Pru av 4

2.2.2 Plant food allergens

Generally, plant proteins, which can cause allergenic reactions, limited to 27 protein families; however, 65% of plant food allergens belong to only 4 families: profilin (actin-binding protein) and Bet v 1 families, prolamin and cupin superfamilies [8] [22]. Common plant food allergens are summarized in Table 3.

Table 3: Common allergic foods from plant source and their major allergens

Food		**Major Allergens**	**Protein Family**	**MW**
Tree nuts	Hazelnut	Cor a 1.04	Bet v 1 family	17.4
		Cor a 2	Profilins	14.1
		Cor a 8	nsLTPs	9.5
		Cor a 9	Legumins	59.1
		Cor a 11	Vicilins	45.1
	Brazil nut	Ber e 1	2S albumins	12.2
		Ber e	Legumins	52.3
	Walnut	Jug r 1	2S albumins	16.4
		Jug r 2	Vicilins	48.3
		Jug r 3	nsLTPs	10*
	Almond	Amandin	Legumins	63.0
	Cashew	Ana o 1	Vicilins	61.8
		Ana o 2	13S globulin	50.5
		Ana o 3	2S albumins	16.3
Peanuts		Ara h 1	Vicilins	67.7
		Ara h 2	2S albumins	19
		Ara h 3&4	Legumins	58.3
		Ara h 5	Profilins	14.1
		Ara h 6	2S albumins	16.9
		Ara h 7		18.4
		Ara h 8	Bet v 1 family	17.0
Soybeans		β-Conglycinin	Vicilins	63.2
		Gly m 3	Profilins	14.1
		Gly m 4	Bet v 1 family	16.8
		Gly m Bd 28k	Vicilins	50.4
		Gly m 1&Bd 30k	Papain-like cysteine proteases	42.8
		glycinin	Legumins	55.7
Celery		Api g 1	Bet v 1 homologues	16.3
		Api g 4	Profilins	14.3
		Api g 5	FAD binding oxidases	9.4
Cereals	Wheat	Tri a 19	Prolamins seed storage proteins	53.0
		Tri a Bd 36K	PR-9 plant protein	8.4
		CM3	α-amlyse/protease inhibitors	18.2
		α - gliadins		36.5
	Rice	RAP		14.5
	Maize	Zea m 14	nsLTPs	9.1
	Rye	Sec c 1	α-amlyse/protease inhibitors	2.9
		Sec c 20	Prolamins seed storage proteins	-
	Barley	Hor v 1&15	α-amlyse/protease inhibitors	16.5
		Hor v 21	Prolamins seed storage proteins	33.2
Mustard		Sin a 1	2S albumins	14.2
		Bra j 1		14.6
Sesame		Ses i 1	2S albumins	17.5
		Ses i 2		17.5
		Ses i 3	Vicilins	67.1

The MW (kDa) referred to calculated mass, the experimental masses were mentioned with *[21][25][26].

The majority of allergens from Bet v 1 family (known also as pathogenesis-related plant protein PR-10 [32]) belong to either Rosaceae fruits such as Mal d 1 in apple, Pru av 1 in cherry, Pru ar 1 in apricot, and Pyr c 1 in pear or Apiaceae vegetables for example Api g 1 in celery and Dau c 1 in carrot [8] [14]. They show often cross-reactivity to pollen [33] and specially to birch pollen. Since they are heat and digestion labile [8], the mild oral allergy syndromes especially itching and swelling of lips are mostly observed reactions [34].

Like Bet v 1 familiy, the profilins are not stable to thermal treatment and enzymatic digestion and their symptoms are restricted to oral allergy syndromes [21]; although, the individual, who are allergic to pollen protein from profilins, are also hypersensitive to a wide range of the other dietary profilin proteins [14]. For example, the allergic persons to grass pollen show cross-reactivity to peanut, tomato, celery, and carrots (due to profiling-specific IgE) [21]; and the allergic individuals to tree pollen are also sensible to celery, carrots, apple, pear, and potato [14]. Allergens of this group were identified in a wide range of fruits and nuts like Pyr c 3 & 4 (estimated to be the same) from pear, Pru p 4 (Pru p 4.01 & Pru p 4.02) from peach, Gly m 3 (Gly m 3.0101 & Gly m 3.0102) from soy, Ara h 5 from peanut, Ana c 1 from pineapple, Pru av 4 from cherry, Api g 4 from celery, Mus xp 1 from banana, Cap a 2 from bell pepper, Cuc m 2 from melon, Lyc e 1 from tomato [26], Cor a 2 from hazelnut, and Lit c 1 from litchi [21].

The prolamin superfamily includes heat- and digestion-resistant proteins with low molecular weight (MW). They have a characteristic eight cysteine skeleton and are rich in α-helices stabilized with disulfide bridges [22]. Despite this structure similarity, they show few sequence identity. This superfamily divided into three groups of major food allergens (prolamin seed storage proteins, 2S albumins and non-specific lipid transfer proteins (nsLTPs)) and less important group of cereal α-amylase/protease inhibitors, which included allergens from cereals such as wheat, barely, rice, rye, and corn [8] and is associated with baker asthma [35]. The major allergens Tri a 19 from wheat, Sec c 20 from rye, and Hor v 21 from barley belong to prolamin seed storage proteins [14], which include 50% of total protein contents in cereal kernels [22]. These sulphur rich proteins [21] are also rich in proline and glutamine, whose combination gave the name prolamine to this family. Many storage proteins of dicotyledon species belong to 2S albumins [14]; and a variety of them are characterised as major allergens

in seed and tree nuts, such as Ara h 2 and 6 from peanut, Jug r 1 from walnut, Ber e 1 from Brazil nut, Sin 1 from yellow mustard [8] [22], Bra j 1 from oriental mustard, and Bra n 1 from rape [14]. The lipid-binding nsLTPs (also known as pathogenesis-related plant protein PR-14) are widely distributed in different plants such as fruits, vegetable, seeds, and nuts, for example Cas s 8 in chestnut, Aspa o 1 in asparagus, Vit v 1 in grape, Zea m 14 in maize, Lac s 1 in lettuce [21], and Cor a 8, which is associated with severe allergic reactions to hazelnuts, belongs to this group [36]. Also the fruit allergens from the Rosaceae family such as Mal d 3 from apple, Pru p 3 from peach [22], Pru av 3 from cherry, Pru d 3 from plum [21] and Pru ar 3 from apricot [14] belong to nsLTP. Characteristic for this group are four disulfide bridges that could be the reason for their thermal and digestion resistance [22]. It is worthy of mention that, Pru p 3 is more stable under acidic rather than neutral condition; it can not refold after heat treatment under neutral conditions, but apparently at pH 3 [37]. The cross-reactivity between pollen and food allergens from this family have been seldom observed [22]. But Pastorello et al. [38] found a very high cross-reactivity among the nsLTP food allergens from the Prunoideae subfamily (peach, apricot, plum and apple), whose similarity is about 95%, and also between maize and peach (despite their botanically unrelated family). The individuals, who suffer from peach allergy, show also allergic reactions to maize; something that is apparently due to structure homology of nsLTP proteins from maize and fruits from Rosaceae family [8]. Pastorello et al. [39] observed high cross-ractivity between Cor a 8 and Pru p 3 as well, but it was denied by Gaier et al. [37]. They could show that Pru p 3 was recognised by polyclonal anti-Mal d 3 antibody from rabbit serum, however not by anti-Cor a 8.

The cupin superfamily, whose proteins include characteristic β-barrel structural domains [21], is classified into vicilin 7S seed storage globulin family and legumin 11S globulin protein family [8] according to their sediment coefficient factors. The 7S globulins are normally trimeric and 11S are hexameric proteins [10]. Allergens belonging to the legumins are rarely glycosylated. Since cysteines failed in vicilins, they can not build disulfide bridges [14] [21]. These allergens could be characterized in many nuts, seeds and legumes; for example major allergens, could be identified as vicilin, are: Ara h 1 in peanuts, β-conglycinin in soybean, Ana c 1 in cashew nuts, Jug r 2 in walnut [10], Len c 1 in lentils, Ses i 3 in sesame [14], and Cor a 11 in hazelnut. Some legumins, characterised as major allergens, are: Cor a 9 in hazelnut,

Cocosin in coconut [26], Ara h 3 and 4 in peanut (assumed to be the same allergen [21]), glycinin in soy, Amandin (almond major protein) in almond [10]. Although there is homology between 11S globulin proteins from different legumes, this sequence homology is more definitive for 7S globulin proteins, where the variable domains are mainly found within the N- and C- terminal regions and not in the inside parts of the sequences; whereas in 11S proteins only the β-polypeptide is conservative and the α-polypeptide is variable [40]. This homologous structure can cause allergic cross reactivity among these nuts; however it is not a guaranty for cross reactivity [4]. Goetz et al. [41] could show strong cross-reactivity of hazelnut to walnut and pecan; and moderate cross-reactivity to cashew, Brazil nut, pistachio, and almond. Also de Leon et al. [42] could evidence the cross-reactivity between peanut and three tree nuts (almond, hazelnut, and Brazil nut). In another study, Koppelman et al. [43] showed the homology between glycinin- and Ara h 3-epitops. This could explain why IgE from peanut-allergic individuals bind to both subunits [21] and often cross-reactivity between soy and peanut were reported [44].

2.2.3 Animal food allergens

The major food allergens from animal origin are milk, egg, and different species of see animals, whose allergens are restricted to even fewer protein families compared to the plant allergens [8] [14]. Common animal food allergens are summarized in Table 4. Generally, three protein families: caseins, parvalbumins, and tropomyosins were reported to be predominated in animal food allergens [22]. However, there are others families, which can prevail the allergens of each animal. For example, the major milk allergens (50-65% of patients) are Bos d 4 (α-lactalbumin), Bos d 5 (β-lactoglobulin), and Bos d 8 (caseins) [3]. α-lactalbumin (ALA) from glycoside hydrolase family 22 is able to bind calcium [22] and constitutes 5% of the whole milk protein [45]. β-lactoglobulin (BLG) from lipocalin superfamily (lipid-binding proteins), whose protein show low sequence similarity [22], is able to bind lipid ligands, which increase its resistance to heat treatment [20]. 10% of whole milk protein consists of β-lactoglobulin. Casein (calcium-binding proteins) accounts for 80% of the milk protein, which is divided into α-casein (42% of whole milk protein), β-casein (28%), and κ-casein (10%). Also the other milk proteins with low concentration trigger allergic reactions in susceptible individuals [3], for example Bos d 6 (bovine serum albumin), which consist

Table 4: Common allergic foods from animal source and their major allergens

Food		**Major Allergens**	**Protein Family**	**MW**
Milk		Bos d 4	Glycoside hydrolase family 22	14.2
		Bos d 5	Lipocalins	19.9
		Bos d 6	Serum albumins	69.3
		Bos d 7	Immunoglobulins	-
		Bos d 8	Caseins	25.1
		Lactoferrin	Transferrins	78.1
Fish	Cod	Gad c 1	Parvalbumins	11.4
		Gad m 1		-
	Carp	Cyp c 1		11.4
	Salmon	Sal s 1		11.9
Crustaceans	Shrimp	Pen i 1	Tropomyosins	34*
		Par f 1		39*
		Pen a 1		32.7
		Met e 1		34*
	Crab	Cha f 1		30.4
	Sessilia	Bal r		38*
	Krill	Eup s 1		38*
		Eup p 1		38*
	Lobster	Pan s 1		31.7
		Hom a 1		32.9
Mollusks	Oyster	Cra g 1& 2		26.9
	Snail	Tur c 1		16.8
		Hel as 1		32.6
		Hel a 1		36*
	Squid	Tod p 1		38*
	Mussel	Per v 1		-
	Abalone	Hal m 1		38*
Egg		Gal d 1	Serine protease inhibitors	22.6
		Gal d 2	Serpins	42.8
		Gal d 3	Transferrins	77.8
		Gal d 4	Glycoside hydrolase family 22	14.3
		Gal d 5	Serum albumins	69.9

The MW (kDa) referred to calculated mass, the experimental masses were mentioned with *. In case of caseins, the MW of β-casein was given [21] [25] [26] [27].

of 1% of whole milk protein, is the other milk allergen with minor designation. Except caseins, which are from coagulum (curd) fraction, the other above mentioned milk allergens are from lactoserum (whey) fraction [45]. It was reported that in many cases, allergic reactions after ingestion of different types of cheese could occur; however it seems that lactic acid fermentation reduced the allergenicity of α-lactalbumin and β-lactoglobulin [18].

Gal d 1 (ovomucoid) from Kazal-type serine protease inhibitor family is the most dominant hen's egg allergen (11% of egg white) [8]. Since this protein is highly glycosylated, it is resistant to enzymatic digestion [22]. The other major allergens of egg: Gal d 2 (ovalbumin), Gal d 3 (ovotransferrin), and Gal d 4 (lysozyme) [14] are also from egg white [5]. The only major allergen from egg yolk is α-livetin, which is identical to chicken serum albumin [18]. The thermo-stabile glycosylated ovalbumin with 54% is the most abundant protein of egg white. Ovotransferrin with 12-13% is a minor allergen, which can bind iron and build disulfide bond, nevertheless it is not very stable to denaturants, but resistant to heating. Lysozyme is only a minor allergen (3.5% of egg white). It shows the highest thermo-stability at pH range 3.5-5. The hens' egg allergens cross react almost only with the egg allergens from other avian [5].

The major seafood allergens belong either to parvalbumin or tropomyosin families. Both of them are resistant to enzymatic digestion and thermal denaturation [22]. Thermostable tropomyosins, with highly conserved domains and identified sequences that lead to cross-reactivity [14], could be found in both important food allergens: mollusc and crustacean. Parvalbumins from calcium-binding EF hand protein family can be found in high concentration (up to 5 mg/g) in white muscles of fish [8]. Although they generally show high resistance to heat treatment and enzymatic and chemical denaturation, but the ability of IgE binding is reduced strongly after processing [46]. Nonetheless it seems that enough epitopes persist after cooking to trigger allergic reactions [20] and the allergenicity risk is not eliminated necessarily after enzymatic digestion. The cross-reactivity between the parvalbumin from fish and amphibians were reported [22].

2.2.4 Background of legislative labelling

The protection of allergic patients is not easy. They can be sensible to different allergens [19], for example the sensitization to major milk allergens (caseins,

α-lactalbumin, and β-lactoglobulin) are closely linked [24]. There is also the risk of cross reactions, e.g. persons with hazelnut allergy suffered often from tree pollen allergy also [34]. The other danger is the presence of isoallergens that result in an increasing number of epitopes. They have the same molecular weight and functional property as the corresponding allergen and about 67% sequence homology, but other isoelectric points. Another problem is the contamination of foods during storage, shipping or manufacturing by undeclared allergens, referred as hidden allergens [11] [18], for example many thickening and stabilizing materials, used by preparing of industrial food products, are proteins from the legume family, which can cross-react with peanuts [19]. Therefore to forewarn the consumer, it was more and more common to use the notion "may contain certain allergen" on the label [1].

In absence of a treatment, the legislation of different countries (for example USA, Canada, Australia, New Zealand, and Japan) recognised the need to improve the labelling policy to ensure the protection of allergic persons [11]. The European Commission had issued the Directive 2000/13/EC on 20 March 2000 about the labelling, presentation and advertising of foodstuffs. In this Directive, all of the member states were obligated to print a detailed list of all ingredients on the food products. This allows the consumer to choice the products in full knowledge; however, in this directive the allergens were not specially mentioned [47]. For better protection of allergic individuals, the European Commission amended this Directive on 10 November 2003 and abolished the 25% rule of 2002, which had allowed some components with less than 25% of compound not to be declared on the label [13]. In Directive 2003/89/EC, it was emphasised again, that appropriate information of consumers regarding to food components is important to achieve a high level of health protection. Therefore all ingredients, which have been intentionally added to the product, have to be listed on the label. This Directive also included a list of 12 common allergens, which are responsible for most allergies or intolerances in consumers (upon the recommendation of the Scientific Committee on Food set up by Article 1 of Commission Decision 97/579/EC). This list consist of food commodities, which shall be referred clearly with their names on the label, and include cereals containing gluten (i.e. wheat, rye, barley, oats, spelt, kamut or hybrid strains), crustaceans, eggs, fish, peanuts, soybeans, milk, nuts i. e. almond (*Amygdalus communis L.*), hazelnut (*Corylus avellana*), walnut (*Juglans regia*), cashew (*Anacardium occidentale*), pecan nut (*Carya illinoiesis)*, brazil nut (*Bertholletia*

excelsa), pistachio nut (*Pistacia vera*), macadamia nut and Queensland nut (*Macadamia ternifolia*), celery, mustard, sesame seeds, and sulphur dioxide and sulphites at concentrations of more than 10 mg/kg or 10 mg/L [48]. Sulphite is not an allergen per se, but can cause release of histamine and is therefore listed. With Commission Directive 2006/142/EC on 22 December 2006, lupine and molluscs were added to this list [49]. In the meantime, the European Food Safety Authority (EFSA) found out that certain compound derived from these listed allergens, in specific cases, do not cause adverse allergic response in sensible people [50]. Therefore the European Commission published on 27 November 2007 in Annex IIIa of the Commission Directive 2007/68/EC a specified list of totally 14 allergens [51]. This list (Fig 2) gives information what has to be declared on the labels of food products and what is excluded, however no thresholds for allergen declaration are given. This leads to zero tolerance, which is not really practical, and unnecessary and vague labeling notations such as "may contain allergens" appear on the food packages.

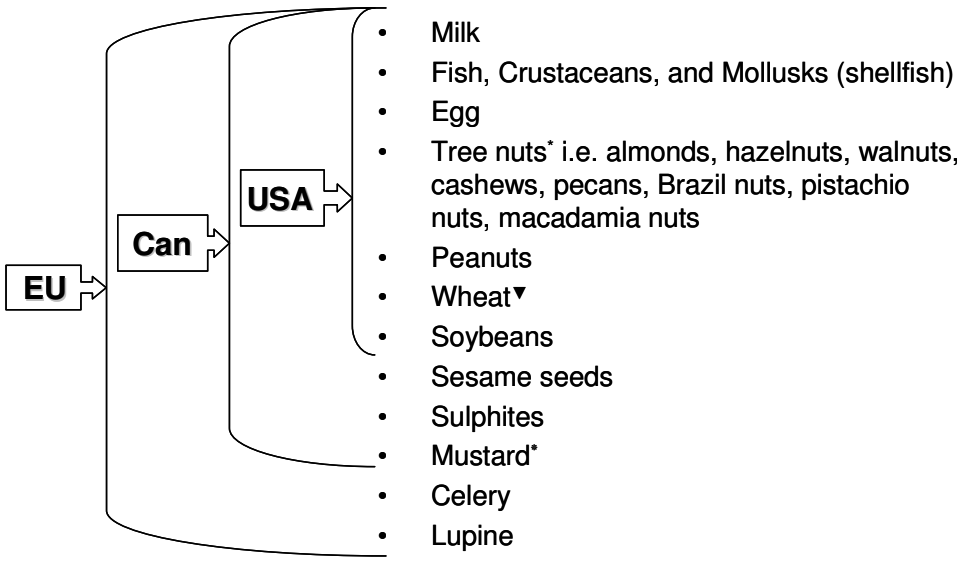

Fig 2: Major food allergen in different countries, which should be declared on the label of foodstuffs according to legislation.
* In Canada, tree nuts include also Pine nuts. ▼ In EU, all cereals, which contain gluten, should be indicated for example rye, barley, oats, spelt or their hybrid strains. * The labelling requirement of mustard will enter into force on August 4th, 2012 in Canada.

In order to get a clear declaration for allergens, the European Voluntary Incidental Trace Allergen Labelling (EU-VITAL) defined a standardized procedure for the labeling of allergens based on the VITAL concept already successfully established in Australia and New Zealand. These action levels (Table 5) define whether labeling is

necessary or not, according to clinical thresholds recommended by European and international scientific expert groups. In other regions such as Japan and Switzerland similar concepts are already established [52]. The European commission will probably introduce action levels for some allergens in 2012.

Table 5: The action levels of major food allergens [mg/kg food] [52]

Allergens	**Action level 1**	**Action level 2**	**Action level 3**
Labeling	not required	required	required as ingredient
Declaration	not necessary	"contains traces of ..."	"contains ... (as ingredient)"
Milk	< 50	50-500	> 500
Egg	< 20	20-200	> 200
Soy	< 25	25-250	> 250
Fish	< 100	100-1000	> 1000
Peanut	< 8	8-80	> 80
Tree nuts	< 10	10-100	> 100
Sesame	< 10	10-100	> 100
Crustacean	< 10	10-100	> 100
Gluten	< 20	20-200	> 200
Celery	< 20	20-200	> 200
Lupine	< 20	20-200	> 200
Mollusks	< 20	20-200	> 200
Mustard	< 20	20-200	> 200
SO_2	< 10	10-100	> 100

The allergens, which have to be declared on the label, are fewer in Canada and USA (Fig 2). In the USA, the Food Allergen Labeling and Consumer Protection Act of 2004, which US Senate legislated as amending the Federal Food, Drug, and Cosmetic Act (21 U.S.C 343), includes eight major food allergens (Fig 2) [53]. In Canada, the first regulatory amendments were published on July 26, 2008 (*Canada Gazette*, Part I) by Health Canada and the second part, CGII (*Canada Gazette*, Part II), on February 16th,

2011, which will come into force on August 4th, 2012. From this date, ten food allergens (Fig 2) will undergo the labelling requirement to provide information for consumer [54].

These regulations promote the development of reliable methods for specific detection and quantification of allergens in food products [1]; something that is not always easy, because often these allergens either occurred in very low concentrations or they are masked by the respective food matrix [13].

2.3 Methods for the detection of allergens in food products

Correct labelling of food products needs different standard analytical methods for the detection of proteins with allergenic potential in foods and foodstuffs. Also reference materials for validation are needed. There is an increasing interest for the approaches that can detect the food allergens faster, cheaper, and more sensitive and specific (lower limit of detection (LOD), better limit of quantification) [17]. Although it is still impossible to determine a generally accepted clinical threshold (since this varies from patient to patient), normally limit of detections between 1 and 100 mg/kg are demanded for the detection methods itself [13] and can be reached.

There are some factors, which affect the quantitative results. One is the choice of an appropriate extraction buffer that affects the extraction yield: the higher extraction efficiency result in more reliable quantification results. The aim of extraction is the solubilisation of target proteins for further use as immunogens or standards for calibration or for analysis. The proteins can be solubilised in different buffers (albumins in aqueous buffers, globulins in saline buffers, and prolamines in a mixture of water and alcohol); therefore, there is not a universal extraction buffer for all food allergens [55]. Adding of additives such as surfactants or reducing agents to extraction buffers can improve the extraction yield [8]. However, it is important that these additives do not manipulate the results, for example using of fish gelatine is not advisable, if the results deal with fish allergens or another allergen, that can cross react with fish allergens. The other factor is the food matrix that can either influence the detection of analyte or make the extraction difficult [11]; an example is chocolate where polyphenols can mask the peanut proteins and reduce the extraction yields down to 50%. To overcome this problem, usage of skim milk powder is often recommended.

The detection methods must not necessarily target the allergen itself; the detection of each component, which is characteristic for the allergenic food, can deduce the

presence of this allergen [8]. Detection of major contents of soluble proteins that likely include the allergenic protein leads to increased sensitivity of the assay and makes the determination of trace amounts of the allergen possible. Recently, in the approaches developed for the detection of allergens in food products, the tendency to use a marker rather than the allergenic protein itself rises. Theoretically, these markers can be any component, which is allergen specific [11]; but these markers are often peptides (normally a conserved part of protein sequence that preferably does not occur in the other proteins). To guarantee the specificity of the marker, extensive database searching is necessary. Using multi-analyte methods, which target various markers and measure different transitions, is also advisable to increase the sensitivity of the detection method.

Polymerase chain reaction (PCR) is almost the only DNA-based method for the detection of food allergens. However, its usage is discussed controversial, because food processing can affect the nucleic acid sequence and the proteins in different ways or during fractionation, DNA may be separated from proteins that can lead to false negative results [13]. The other limitation of PCR is that some allergenic food commodities have no DNA, for example egg white, which includes major egg allergens, has no DNA. Therefore, PCR is not the method of choice for allergen analysis; nevertheless, it is a good choice for the detection of allergens in commodities that have no high concentrations of proteins. An example is celery, whose protein is not heat stable, and therefore there is currently no enzyme-linked immunosorbent assay (ELISA) for its identification, but two PCR-based detection methods [56].

The PCR amplifies a specific part of allergen DNA and produces a huge number of copies. To ensure the specificity, the used primers serve as starting points for synthesis of the specific DNA [11]. This synthesised DNA can be detected by either gel electrophoresis or ELISA. Detection by gel electrophoresis results in a qualitative method; but with using PCR-ELISA or fluorescence monitoring real-time PCR, both detection and quantification is possible [1]. In real-time PCR, the amplified DNA is detected after each cycle, in contrast to PCR combined with gel electrophoresis, where the detection is performed at the end of the procedure.

In general, the approaches for the detection of proteins include almost exclusively immunoanalytical methods, which use specific antibodies for the detection of target proteins such as qualitative methods immunoblotting and rocket immunoelectrophoresis or quantitative approaches like ELISA, radio-allergosorbent test, enzyme allergosorbent

test, and radioimmunoinhibition assay [13]. There are two classes of immunoassays: direct and indirect. The first one measure directly the actual antibody-allergen bindings; the second one measures the antibody-allergen reactions indirectly for example with a second anti species antibody, which recognizes the first antibody.

The production of suitable antibodies is an important step in development of immunoassays [23]. These antibodies, which should be capable to recognize the proteins even in a complex mixture of different components [55], are produced in different mammals, which are immunised with a purified protein or a protein mixture extracted from a food commodity [8]. The antibodies produced by using a crude protein extract for immunisation of mammals are capable to detect all kind of the respective proteins [23].

Among the above mentioned methods, only ELISA and PCR are used currently for routine analysis in food industry, and the usage of the other ones is restricted only to research purposes [17]. Since the immunobased methods are rapid, sensitive and selective, they have used in recent years for developing several test kits. Especially fast ELISA test kit and immunochromatographic tests make the food monitoring for allergen ingredients or cross-contamination easy [1]. However these immunochemical methods have some drawbacks: 1) if the structure of target protein was changed (for example due to heating or proteolytic treatment), the used antibodies may not detect the protein anymore which can lead to false negative results; 2) because of cross-reactivity, a false-positive result can be achieved; and 3) generally, simultaneous detection of several allergens is difficult, for example: for the detection of each food commodity, a single ELISA kit is necessary. Therefore, alternative methods, which offer comparative and complementary techniques, are demanded.

Mass spectrometry (MS) is an analytical technique that is not based on antibodies and measures the different components by converting them to charged ion. MS is one of the methods with very high specificity, which can provide quantitative information of analytes with high sensitivity (detection of trace amounts is possible) [57] and has the advantage that makes the simultaneous detection of multiple allergenic proteins possible [3]. Furthermore, MS combined with high resolution separation devices makes an unambiguous detection [11] and quantification possible, without the need of purification of proteins and peptides prior to determination [58].

Nevertheless each of these techniques (immunoassays or MS) and different strategies (detection of either target allergen or a specific protein or markers) are suitable for different cases and therefore all of them have to be implemented and compared to decide which of them are the best one for the intention. Here an immunobased method (ELISA) and a MS-based technique (LC-ESI-MS/MS), which were used in this work, are introduced in detail.

2.3.1 ELISA

ELISA has become popular in the 1980s and is currently the most used detection method in food industry because of its wide range of possibility and its high analytical potential. The reaction is specific, the method is sensitive and has high screening capacity [8]. It can be design as field-portable assay and normally by changing the hardware no long sample preparation is needed [14]. The specificity of this approach is guarantied by the selection of appropriate antigen-specific antibodies [8]. Also suitable materials for coating are needed. The coated substrate shall not react with the other elements of the ELISA; therefore, the blocking is an important step during method development [2]. Beside antibodies and hardware, appropriate amounts of purified allergen and a stable enzyme detection system are necessary to build a high quality ELISA system [14].

Nowadays, a variety of ELISA kits are commercial available and many of them are capable to simultaneous detect different allergens [11]. Generally in ELISA one component (antibody or allergen) is immobilised. To simplify the detection of bound components, one component is conjugated to an enzyme that converts a substrate to a coloured product. Alkaline phosphatase and horseradish peroxidase (HRP) are the commonly used enzymes in ELISA. Since HRP does not cost much, it is used more often. It can either produces light after reaction with chemiluminescent substrates, or restructure the chromogenic substrates such as *o*-phenylenediamine (OPD) or 3,3´,5,5´-tetramethylbenzidine (TMB) into colored products. TMB, which (unlike OPD) is not a hazardous material, forms a blue product in solution after reaction with peroxidase enzymes HRP. The color change can be measured after enzyme destruction at a specific wavelength [14]. Two ELISA techniques can be distinguished for the quantification of proteins in food analysis: sandwich and competitive format [1].

2.3.1.1 Competitive ELISA

This ELISA format is commonly used for the detection of small molecules and based on the competition of the molecules present in samples and immobilised ones for antibody [14]. In competitive ELISA, the solid surfaces can be coated with e.g. allergen or hapten. After washing the plate and blocking, in direct competitive ELISA (Fig 3 a), the inhibitors and anti molecule enzyme-labelled antibody are filled into each well. Since the molecule present in samples competes for antibody, the amount of antibody bound to immobilised molecules is reduced, which results in decreased possibilities to built further coloured derivates; therefore the absorbance of formed coloured products is inversely proportional to the concentration of molecules in samples [13]. In case of unavailability of enzyme-labelled analyte-specific antibody, indirect detection can be used (Fig 3 b). At first inhibitors and then an anti molecule antibody is added to the immobilised molecule. In the second step, the secondary enzyme labelled antibody is added for further visualization. Standard curves are created by using the four parameter logistic equation [1].

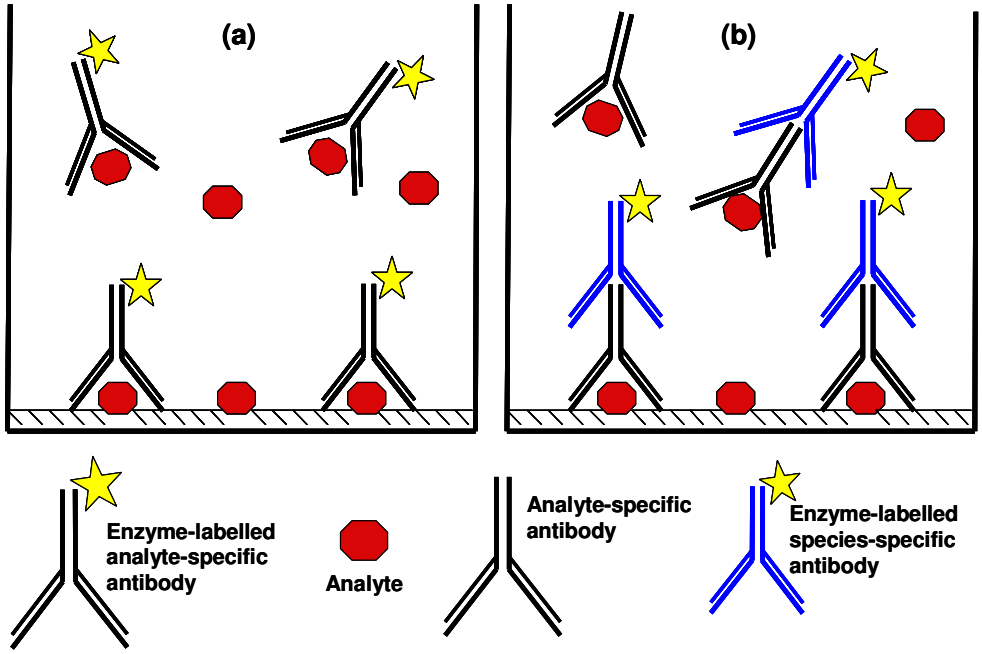

Fig 3: Competitive ELISA
(a) Direct detection using labelled analyte-specific antibody
(b) Indirect detection with labelled species-specific antibody [2]

There are some important factors for the development of competitive ELISAs. For the coating of solid surface, a large quantity of substance is necessary. This substance must be capable to bind to the solid surface with additional remaining free

epitopes for the binding of the antibody. And last, the antibody must recognize the bound and unbound molecules with the same efficiency [23].

2.3.1.2 Sandwich ELISA

This form of ELISA is the common immunoassay type used for the detection of potential food allergens [13]. Its only drawback is the need of a high and continuous amount of purified antibody [14]. For the immobilization of capture antibody on a solid phase, the wells of microtitre plates or multiple well strips are coated with one analyte-specific antibody [1]. Then, incubation with the sample follows. The unbound proteins are washed away and with the second analyte-specific enzyme-labelled antibody the sandwich format is created. Finally visualization with a colorimetric reaction is performed (Fig 4 a). The second antibody has to be analyte-specific and shall not attach to the same epitopes as the immobilised antibody [8]. Again if no enzyme-labelled analyte-specific antibody is available, the indirect procedure can be used (Fig 4 b) as already explained for competitive ELISA. But in this case the two used analyte-specific antibodies must be derived from different species [1].

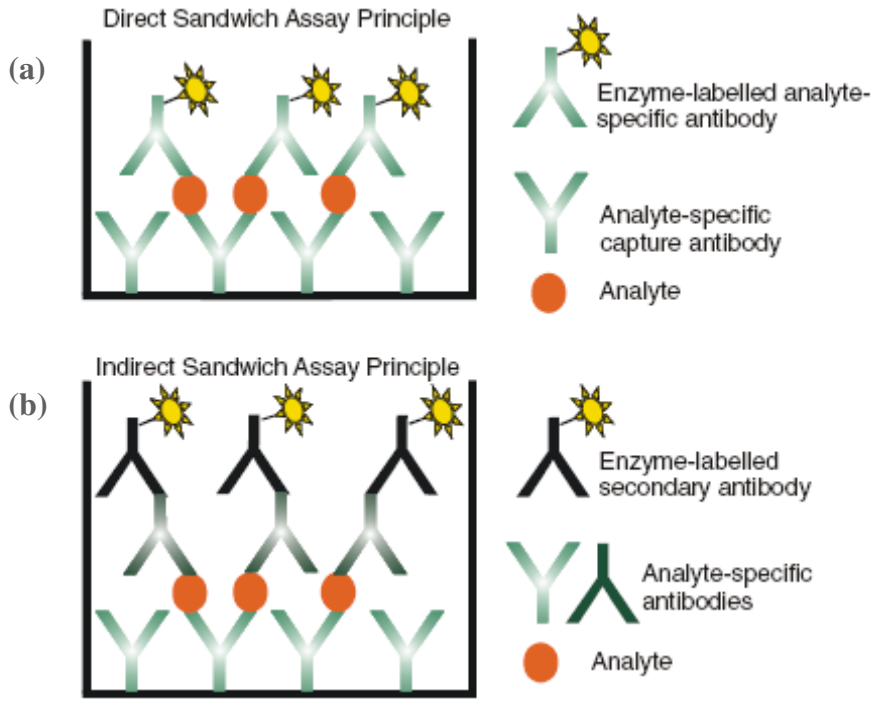

Fig 4: The schematic diagram of sandwich ELISA
(a) Direct detection using an enzyme-labelled analyte-specific antibody
(b) Indirect detection with an enzyme-labelled species-specific antibody [1]

The colorimetric detection is performed in the same way as for the competitive format. In case of the sandwich format, the absorption is directly proportional to the concentration of the allergen [13].

2.3.2 Mass spectrometry

Mass spectrometry, primarily used for the determination of the mass of molecules, nowadays plays a central role in almost every area of science because of its ability to measure at high level of molecular specificity and detection sensitivity [57]; however, it has to be considered that in MS analysis, the sample preparation is a decisive step for sensitivity [59]. MS is also a central analytical technique for the study of biomolecules [60]; especially in the field of protein studies, it has been applied in the last several decades in a wide range of application beside the molecular weight determination of whole protein [61], for example characterisation of post-translational modifications, ligand binding, sequencing of enzymatic digested peptides for structural characterization, protein conformations (native, denatured, folding intermediates), and protein–protein interactions [58]. The disadvantage of this method is the need of expensive special equipment; its advantage is the possibility of simultaneous detection of different allergens in a single run [11].

Instead of measuring the molecule itself, the ions are measured by MS analysis, because the manipulation of the direction and motion of ions is easily manageable by applying electric and magnetic forces. The basic concept of MS, which involves three main steps, is demonstrated in Fig 5. The first step is the ionisation in an ion source, where an electron or proton is removed from the analyte and the ion is produced. The fragmentation of these ions and building of product ions is also possible. In the second step, these ions are separated and measured with a mass analyser according to their mass to charge *(m/z)* ratio. In the last step, the obtained data is amplified and displayed in form of mass spectra [57].

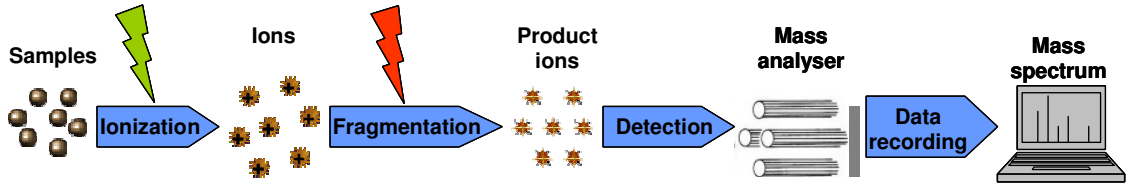

Fig 5: Basic concept of MS

2.3.2.1 Liquid chromatography

Liquid chromatographic separation systems are the common used methods for the analytical and preparative separation of food proteins that are often coupled to a UV or fluorescence detector [58]. In high-performance liquid chromatography (HPLC) using smaller particles as stationary phase results in lower plate height values and enhanced chromatographic separation efficiencies. Recently commercialised ≤ 2 µm particles can shorten the run time and improve the separation of analytes and matrices. Although the MS instruments have a high selectivity, without sufficient sample cleanup and chromatographic separation, the matrix components can impair the accuracy of quantitative method. These co-eluting components can either affect the evaporation of the droplets or compete for electrical charge against the analytes, which leads to ion suppression [62]. This can be solved by coupling of a separation technique such as gas chromatography (GC), HPLC, or capillary electrophoresis (CE) to MS [57]. Whatever the separation approach is, it adds an additional dimension to the analytical measurement [63]. For example, with hyphenation of MS to HPLC, the background signals can be removed and the concentration of analytes increase. This results in higher signal-to-noise ratios. It has to be mentioned that in this case the complete chromatographic resolution is not necessary and time-consuming purification and fractionation steps can be avoided [58].

2.3.2.2 Electrospray ionisation

Ionisation is the first and most challenging step in the MS analysis. Different type of ion source have become available over the years [57]; nonetheless since the techniques for soft ionisation without excessive fragmentation were lacking, MS analysis was restricted for a long time only to small and thermostable elements [60]. The development of soft ionization techniques such as electrospray (ESI) and matrix-assisted laser desorption (MALDI) changed the situation and resulted in the increasing use of MS for the analysis of large, non-volatile, and chargeable molecules such as proteins [63]. Because of the sensitivity of these methods, their high mass range and their capacity to analysis complex mixture without separation, and also because of their capability to extend by collision-induced fragmentation, these methods were used for characterisation of large biomolecules to get information about the molecular structure and post-translation modifications [58].

ESI is currently the most universal and versatile ionisation technique, because a wide range of analytes can be ionized by ESI. Its only restriction is that the compound shall be sufficient polar to allow the attachment of the charges; for example proteins, oligonucleotides, sugars, and polar lipids fulfil this requirement [59]. ESI showed also the most successful interface for LC/MS applications. A schematic of a typical ESI is shown in Fig 6. The analytes are injected directly from either an infusion pump or HPLC into the ionisation source through a stainless steel capillary tube, whose tip held a high voltage comparing to the electrode, which surrounds the area with atmospheric pressure. This potential difference causes the production of an electric field, which converts the injected analytes to small charged droplet [57]. The desolvation of these droplets is achieved by either heating, differential pumping [58] or employing an uncharged countercurrent gas flow such as rare gas, N_2 or pure air [64]. The eluents are often aqueous buffers containing organic solvent and low molarities of weak volatile acid or base to promote the ionisation of the samples. The in atmospheric pressure produced ions enter the high vacuum mass analyser through an orifice. With interfacing the electrospray to different mass analyzers such as time-of-flight (TOF), quadrupole mass filter, ion traps, and Fourier transform ion cyclotron resonance, accuracies of 0.001% in mass determination can be achieved [58], however the combination of ESI to a quadrupole mass analyser is the most successful one [57].

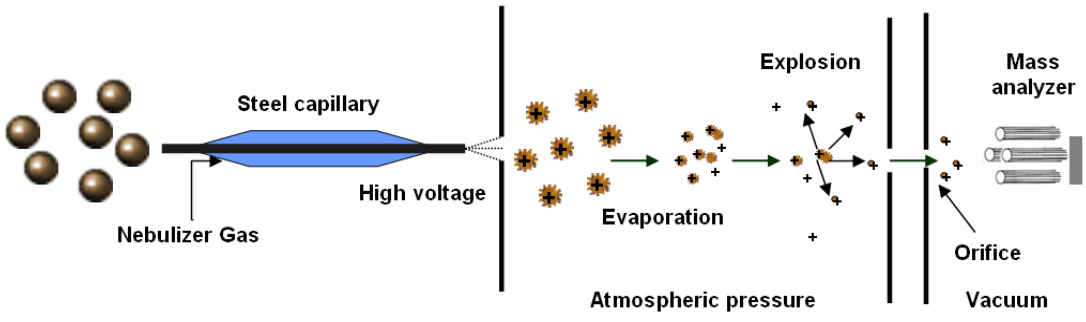

Fig 6: Basic component of electrospray ionization

2.3.2.3 Quadrupole mass analyser

The mass analyser is the heart of a mass spectrometer. Tandem MS capability, low cost and small size are the desirable characters of a mass analyzer. There are different types of mass analyser; however, the quadrupole devices are the common used

types. A quadrupole (Q) consists of four metal rods, where every apposite pairs is electrically connected to direct current (dc) and radio frequency (rf) power supplies (Fig 7). They produced a high frequency oscillating electric field, along which the ions have to pass with vibratory motions. It can be adjusted by applying certain dc and rf potentials, which ions with a specific *m/z* can pass this field. These potentials are changed to obtain the mass spectrums, but their ratio is constant [57]. Depending on the polarity-adjustment of the instrument, both positively and negatively charged ions can be detected [58].

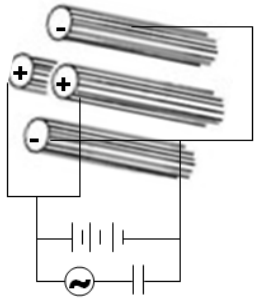

Fig 7: Quadrupole mass analyser

2.3.2.4 Tandem mass spectrometry

In the recent years, tandem mass spectrometry has been used more and more for the identification and quantification of different compounds in complex mixtures. It is related to the coupling of two or more stages of mass analysis (MS^n) [57]. Each stage provides an additional dimension of isolation, selectivity and structural information. If quadrupole and sector instruments are combined, the procedure occurs subsequently in the following spaces of the device, which are called tandem-in-space (Fig 8); TOF and quadrupole are two examples of this type tandem MS. In the other technique (tandem-in-time), all of the processes are performed sequentially in the same region. Ion traps (IT) belong to this group [63]. In MS/MS, the accurate masses of compounds can be determined and a precursor ion at a defined *m/z* can be selected for fragmentation in the collision cells. The generated product ions are trapped and scanned at high sensitivity in the detector [3]. With analysis of these fragments, the detailed structure of peptides can be inferred. The MS/MS in product ion mode can be used to determine the amino acid sequence of the peptides, in SRM mode for quantitative analysis with very high sensitivity and selectivity [60]. In MS/MS, for accurate fragment information from a certain precursor, ion activation and dissociation is necessary. They are used to increase

the number of precursor ions with energies over the dissociation threshold. The collision induced dissociation (CID), also known as collisionally activated dissociation (CAD), is the common used technique for ion activation and dissociation [57].

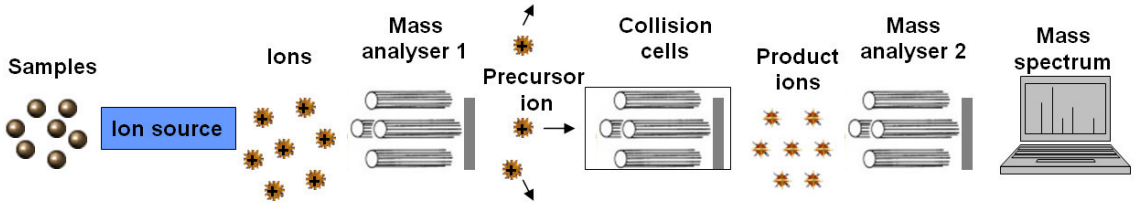

Fig 8: Basic principle of tandem-in-space MS

The combination of LC with tandem mass spectrometry is easily possible [58]. The commonly used tandem mass spectrometers for the detection of trace amounts of elements are triple quadrupole instruments (QqQ), ion traps (IT), and quadrupole-time-of-flight (Q-TOF); among them, QqQ and QIT have high sensitivity, short dwell time, and wide linear range, if they are operated in selected reaction monitoring (SRM) mode. Therefore, they are the most suitable one for the quantification and screening of target and multi-target analysis [62].

2.3.2.5 Quantification

The quantification at protein level can be performed with either external or internal standards [17]. The external standardization is made using calibration curves, where the measured intensities are plotted versus the concentrations of the analytes. To enhance the accuracy of these curves, multiple determinations are often performed. The standard curves are normally linear over a wide range of concentrations. By internal standardization, the standard at a known concentration is added to the sample, before any clean-up step to make the same change of the concentration in sample and standard. Since the isotopomers have the identical ionization efficiency but different mass as the target analyte, they can be used as special internal standards [63].

For quantification at peptide level, three methods were described: tagging, isotopically labelled synthetic peptides, and a label free method. All of these methods can be used for relative or absolute quantification. In tagging methods, the protein or peptide labelled with heavy (^{13}C) or light (^{12}C) stable isotopes and can act as internal standard. They are classified into metabolic, chemical, and enzymatic labelling; and are appropriate for biomarker identification. The next method uses as reference isotope-labelled synthetic peptides, which include ^{13}C or ^{15}N, and therefore differ to the

endogenous peptides with a certain mass. In this method, the choice of peptides is essential; it must be specific to the target protein, to avoid false positive results. The peptides have to be stable in solution. This means that some amino acids, such as methionine and cysteine, which can be oxidised, shall not be included in the peptide. In the recently developed label free method, the sample preparation is simplified and isotopes are not used. The quantification is performed either with the measurement of spectra counting or on the ion signal intensity [17].

2.3.2.6 State of the art: Bottom-up approaches for food allergen detection

For protein identification by MS, two approaches are used: bottom-up and top-down. In top-down approaches, the intact proteins, solubilised from food commodities, are sprayed directly through electrospray and analysed by MS [64]. The more specific bottom-up approach comprises the digestion or cleavage of the protein with an enzyme such as trypsin or chemical cleavage respectively, followed by mass spectrometric analysis of the derived peptides. Each of these peptides that specific for the target allergen is can be used as marker. These marker-peptides shall not be modified during the processing or not affected by Maillard reaction, therefore also intensive database search is essential. The accuracy of mass detection for small molecules is more precise, therefore these measurements are generally more effective rather than top-down analysis, whose analytical throughput and efficiency are still limited because of large-scale proteome analysis [3]. Additionally in peptide-scale MS, the determination is independent of the 3D structure of allergens, and so the detection even after food processing is possible [17]. Many food products can or do contain different allergens. Therefore the needs of simultaneous monitoring of various components result in to developing multianalyte methods, which can target several markers from different proteins in a single run [62].

The bottom-up approach has been recently utilized more and more for the detection of different food allergens. The developed methods either used the peptides of a single protein for the detection of a food commodity (e.g. confirmation of peanut protein in chocolate using Ara h 1 [65], and detection of κ-casein in dairy products [66]) or they used the peptides of several proteins for the identification of the presence of a food commodity or divers food commodity, respectively. For example, Chassaigne et al. selected peanut-specific sequence tags from three major peanut allergens Ara h 1, Ara h 2 and Ara h 3 as markers. The peptides were obtained from tryptic digestion of peanut

samples and identified by nano-ESI-Q-TOF-MS/MS [67]. Almost contemporaneously Careri et al. used the peptides from tryptic digestion of only two major peanut allergens Ara h 2 and Ara h 3/4 as biomarkers for the quantitative detection of peanut by an LC-ESI-MS/MS method [68]. Only one peptide (SPDIYNPQAGSLK) was used in the both methods and other markers are not similar.

In the field of mass spectrometric detection of milk allergens, the literature is scarce. Recently published methods used ESI-Q-TOF-MS for the identification of peptides only from caseins. They rely on a comparison of retention times in LC-MS full scan with compound confirmation by data-dependent MS/MS product ion scan of standard and samples. In this case the risk of negative influence of unknown co-eluting matrix components on the signals of chosen diagnostic peptides can not be excluded. We could develop a MS-based method in SRM mode (with ESI-Q-MS/MS) for the detection of milk using the peptides derived from four milk major allergens caseins, α-lactalbumin, and β-lactoglobulin [3]. Using several peptides from different allergens of a food commodity makes an unambiguous determination of milk possible and increase the specificity of the method.

To the best of our knowledge, only two multi-allergen methods for the simultaneous analysis of allergens from different food commodities (including hazelnut) were published. Bignardi et al. developed a method using LC-ESI-LIT-MS/MS for the analysis of five nut and peanut allergens (Ana o 2 from cashew, Ara h 3/4 from peanut, Cor a 9 from hazelnut, Jug r 4 from walnut, and Pru 1 from almond). All of these allergens belong to 11S globulin family [69]. Also Heick et al. investigated the detection of eight allergens from seven food commodities (prunin from almond, ovalalbumin from egg, Cor a 9 from hazelnut, α-casein from milk, Ara h 1 and 3/4 from peanut, glycinin from soy, and Jug r 1 from walnut) with liquid chromatography and triple-quadrupole tandem mass spectrometry [70]. During this thesis, a novel MS approach (a LC-MS/MS method in SRM mode) was developed for the specific detection of hazelnut using eight peptides from three major hazelnut allergens (Cor a 8, Cor a 9 and Cor a 11). The specificity of selected peptides was verified not only by BLAST search but also with analysing the different nuts with this method; therefore these peptides can be used as markers in the multi-analyte method for the detection of hazelnut in food samples [4].

3 Results

3.1 Commercialized rapid immunoanalytical tests for determination of allergenic food proteins: an overview

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REVIEW

Commercialized rapid immunoanalytical tests for determination of allergenic food proteins: an overview

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Abstract Food allergies have become an important health issue especially in industrialized countries. Undeclared allergenic ingredients or the presence of "hidden" allergens because of contamination during the food production process pose great health risks to sensitised individuals. The EU directive for food labelling lists allergenic foods that have to be declared on food products by the manufacturers. The list includes gluten-containing cereals, crustaceans, eggs, fish, peanuts, soybeans, milk, various nuts (e.g. almond, hazelnut, and walnut, etc.), celery, mustard, sesame seeds, lupin, and molluscs. Reliable methods for detection and quantification of food allergens are needed that can be applied in a fast and easy-to-use manner, are portable, and need only limited technical equipment. This review focuses on the latest developments in food allergen analysis with special emphasis on fast immunoanalytical methods such as rapid enzyme-linked immunosorbent assays (ELISA), lateral-flow immunochromatographic assays (LFA) and dipstick tests. Emerging technologies such as immunochemical microarrays and biosensors are also discussed and their application to food allergen analysis is reviewed. Finally, a comprehensive overview of rapid immunochemical test kits that are currently available commercially is given in tabular form.

Keywords Immunoassay · Strip tests · Microarray · Biosensors · Surface plasmon resonance · Resonance-enhanced absorption

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Introduction

Food allergies have become an important health problem all over the world. Adverse health effects due to allergic reactions to foods or food ingredients occur in about 1–3% of the population and about 4–6% of children (including food intolerances) [1]. Even the intake of minute amounts of food allergens can cause allergic reactions in sensitised individuals. A wide range of allergic symptoms are observed, for example digestive disorder, respiratory symptoms, circulatory symptoms, and skin irritations. However, for some allergic individuals contact with specific food allergens can lead to life-threatening situations (anaphylactic shock). It is important to distinguish between food intolerances and food allergies. Reactions due to food intolerances do not involve the immune system whereas allergies involve an abnormal immunological reaction to a food or certain food ingredient, which provokes the production of allergen-specific IgE antibodies (or IgA and T cells) against a food allergen. In this context, celiac disease, also known as gluten-sensitive enteropathy, is worth a special mention. Gluten (a specific protein fraction of, e.g., wheat and rye) is known to cause damage to the absorptive epithelial cells of the small intestine, leading to malabsorption of nutrients. Although celiac disease is not classified as an allergy, an immunological aspect does exist, which suggest that the disease is at least partly mediated by IgA and cytolytic T Cells [2].

A vast number of food materials have been identified as potentially allergenic; of these only eight account for more than 90% of all food allergies [3]. For an allergic consumer it is particularly important to know the exact composition of foodstuffs, especially regarding the presence of potentially allergenic ingredients or contamination. In order to protect consumers, the European Commission has issued Directive EC 2003/89 as an amendment to EC Directive 2000/13 for

ingredient declarations on foodstuffs [4, 5]. Annex IIIa of this guideline includes a list of food ingredients and products thereof classified as being possible allergens or leading to possible intolerance, e.g. cereals containing gluten, crustaceans, eggs, fish, peanuts, soybeans, milk (including lactose), nuts (almond, hazelnut, walnut, cashew, pecan nut, Brazil nut, pistachio nut, macadamia nut, and Queensland nut), celery, mustard, sesame seeds, and sulfur dioxide and sulfites. Directive EC 2003/89 was issued on 11–25–2005 and requires that any of the twelve described potentially allergenic ingredients are labelled even if they make up less than 25% of the food product. In 2006, the list of allergens in Annex IIIa was expanded to include another two food ingredients, namely lupin and molluscs [6].

An additional problem is contamination of originally non-allergenic food products with traces of food allergens through the production process, so-called "hidden" food allergens. As a precaution, food manufacturers often label their food products with "may contain" a certain allergen. This drastically limits the variety of food products suitable for allergic consumers. It is not only in the interest of the allergic consumer, but also in the economic interest of food manufacturers to be able to guarantee the absence of allergens in food products. Therefore, analytical tools are necessary for reliable and accurate detection and quantification of allergens in foodstuffs.

The methods currently used for the detection of potential allergens in foods target either the allergen itself or a marker that indicates the presence of the offending food. Such food markers are usually proteins specific for the investigated allergenic food or characteristic DNA fragments. Poms et al. [3] gives a comprehensive overview of the methods currently used for allergen analysis in foods. Protein-based methods include immunoblotting, rocket immunoelectrophoresis (RIE), radio-allergosorbent test (RAST), enzyme allergosorbent test (EAST), radioimmunoinhibition assay (RIA), enzyme-linked immunosorbent assay (ELISA), and liquid chromatography–tandem mass spectrometry (LC–MS^n). Immunoblotting and RIE only offer qualitative or semi-quantitative results whereas RAST, EAST, ELISA, and LC–MS^n are quantitative methods. DNA-based approaches involve the amplification of a specific DNA fragment of the allergen gene or food-specific protein gene through polymerase chain reaction (PCR). The amplified DNA-fragments are usually detected by gel electrophoresis or ELISA. Superior quantitative results can be achieved by employing real-time PCR or PCR–ELISA [3, 7].

The threshold dose above which sensitised individuals show allergic reactions varies widely among individuals and is, therefore, difficult to assess. There is, however, general agreement that the detection limits of analytical methods developed for different food products need to be somewhere between 1 and 100 mg of allergenic protein per kg food (ppm) [3]. Most commercially available test kits for the detection and quantification of allergens in food products involve ELISA or PCR technology, which already meet this criterion and have limits of detection (LOD) in the range of 0.1 to 10 mg kg^{-1} (ppm). The declaration of LODs, however, differs between the test kit suppliers and refers either to the allergic protein or to the amount of allergenic food in a certain food product.

Most of the methods developed for allergen analysis in food are time-consuming, labour-intensive, and mostly need expensive laboratory equipment (such as a micro-plate reader or thermocycler) and well-trained people to operate them. Therefore, great emphasis has been put in the development of analytical methods that can be applied in a fast and easy-to-use manner in order to enable legislation, and maybe also consumers themselves, to monitor food products for the presence of allergenic food ingredients or hidden allergens, and to offer manufacturers a tool for on-site check during food production, to avoid cross-contamination.

This review focuses on rapid immunochemical methods for the determination of allergens in food, for example rapid ELISAs using microwells, ELISAs in lateral flow (LF) device or dipstick formats, immunochemical microarrays, and biosensors. Recent developments in both academic and commercial research are highlighted. Special emphasis is given to the major food allergens that are listed in Annex III of the EU food labelling directive [4–6] and on commercial available rapid tests. Sulfur dioxide and sulfite are not included in the discussion, because they are not protein-based food allergens, and are only listed in the EU labelling directive because of their association with asthmatic reactions in sulfite-sensitive individuals.

Rapid ELISA

In the 1980s, ELISA technology had its breakthrough in biochemical and biomedical applications because of its high specificity, sensitivity, and simple sample handling. Its high potential for standardisation and automation enables high sample through put which is essential for screening purposes. ELISA is currently the most widely used analytical technique for routine measurements and screening of allergens in food. Easy-to-use and more or less rapid test kits have become commercially available for a great variety of food allergens with LODs ranging from 0.05 to 10 mg kg^{-1}, depending of the allergen and the food matrix (Table 1). ELISAs test for the presence of allergens or specific food marker proteins by using specific antibodies that are usually raised in mammals such as mice, rabbits, goats, and sheep. However, antibodies generated in mammals are harvested from blood, which implies frequent bleeding or even killing of the immunised animal. Because

Table 1 Commercially available rapid immunochemical test (November 2008)

Allergenic food	Targets	Test format	LOD (LOQ)	Validation	Testing time	
Cereals with gluten	Gliadin, secalins, hordeins	ELISA	2 (5)[f]		30 min	RIDASCREEN FAST Gliadin (R-Biopharm,)
	Gliadin	LFA	2.5 (only qual.)[f]		5 min	RIDA QUICK Gliadin (R-Biopharm)
	Gluten	LFA	<20[a,f]		< 10 min	RAPID 3-D Gluten test kit (Tepnel,)
	Gliadin	ELISA	<10 (only qual.)[f]		30 min	Alert for Gliadin Allergen (Neogen,)
	Gliadin	ELISA	n/s (5)[f]		30 min	Veratox for Gliadin Allergen (Neogen)
	Gliadin	ELISA	n/s (2.5)[c]		n/s	Gliadin assay (ELISA Systems,)
	Gluten	LFA	10 (only qual.)		10 min	EZ Gluten Test (ELISA Technologies,)
	Gluten	dipstick	10–20 (25) (semiquant.)		6–15 min	HAVen GLUTEN FlowThrough Test (Hallmark,)
Crustaceans	n/s	LFA	5 (only qual.)[a]		<10 min	RAPID 3-D Shellfish test kit (Tepnel)
	Tropomyosin	ELISA	n/s (0.05)[c]		60 min	Crustacean Residue ELISA (ELISA Systems)
Eggs	Egg-white protein (ovalbumin, ovomucoid, ovotransferrin, lysozyme)	ELISA	0.6 (1)		35 min	RIDASCREEN FAST Egg (R-Biopharm)
	Egg white protein	LFA	n/s[a]		<10 min	RAPID 3-D Egg test kit (Tepnel)
	Egg protein	ELISA	<5 (only qual.)		30 min	Alert for Egg Allergen (Neogen)
	Egg protein	ELISA	n/s (2.5)		30 min	Veratox for Egg Allergen (Neogen)
	Egg-white protein	ELISA	n/s (1)[c]		35 min	Egg Residue ELISA (ELISA Systems)
Peanuts	Ara h1	ELISA	0.15 (0.25)	AOAC-RI 2003	30 min	RIDASCREEN FAST Peanut (R-Biopharm)
	Ara h1	LFA	5 (only qual.)		10 min	RIDA QUICK Peanut (R-Biopharm)
	Peanut protein	LFA	1 (only qual.)[a]	JRC-IRMM 2006	<10 min	RAPID 3-D Peanut test kit (Tepnel)
	Peanut protein	LFA	<5 (only qual.)	JRC-IRMM 2006	10 min	Reveal for Peanut Allergen (Neogen)
	Peanut protein	ELISA	<5 (only qual.)		30 min	Alert for Peanut Allergen (Neogen)
	Peanut protein	ELISA	n/s (2.5)	AOAC-RI 2003	30 min	Veratox for Peanut Allergen (Neogen)
	Ara h1, Ara h2 and others	ELISA	n/s (1)[c]		35 min	Peanut Residue ELISA (ELISA Systems)
Soybeans	Soy protein	ELISA	<5 (only qual.)		30 min	Alert for Soy Flour Allergen (Neogen)
	Soy protein	ELISA	n/s (2.5)		30 min	Veratox for Soy Flour Allergen (Neogen)
Milk	Casein	ELISA	0.12 (0.5)		30 min	RIDASCREEN FAST Casein (R-Biopharm)
	Casein	LFA	n/s[a]		< 10 min	RAPID 3-D Casein test kit (Tepnel)
	Casein and whey	LFA	<5 (only qual.)[d]		5 min	Reveal for Total Milk Allergen (Neogen)
	Milk protein	ELISA	<5 (only qual.)[e]		30 min	Alert for Total Milk Allergen (Neogen)
	milk protein	ELISA	n/s (2.5) [e]		30 min	Veratox for Total Milk Allergen (Neogen)
	β-lactoglobulin	ELISA	n/s (0.1)		45 min	Beta Lactoglobulin Residue ELISA (ELISA Systems)
	Casein	ELISA	n/s (1)[b]		45 min	Casein Residue ELISA (ELISA Systems)
Nuts						
Almond	Almond protein	ELISA	0.17 (0.25)		30 min	RIDASCREEN FAST Almond (R-Biopharm)
	Almond protein	LFA	1 (only qual.)		<10 min	RAPID 3-D Almond test kit (Tepnel)
	Almond protein	ELISA	<5 (only qual.)		30 min	Alert for Almond Allergen (Neogen)

Table 1 (continued)

Allergenic food	Targets	Test format	LOD (LOQ)	Validation	Testing time	
	Almond protein	ELISA	n/s (2.5)		30 min	Veratox for Almond Allergen (Neogen)
	Almond protein	ELISA	n/s (0.5)		35 min	Almond Residue ELISA (ELISA Systems)
Hazelnut	Hazelnut protein	ELISA	0.15 (0.25)	BVL 2006	30 min	RIDASCREEN FAST Hazelnut (R-Biopharm)
	Hazelnut protein	LFA	5 (only qual.)		10 min	RIDA QUICK Hazelnut (R-Biopharm)
	Hazelnut protein	LFA	1 (only qual.)[a]		<10 min	RAPID 3-D Hazelnut test kit (Tepnel)
	Hazelnut protein	ELISA	n/s (2.5)		30 min	Veratox for Hazelnut Allergen (Neogen)
	Hazelnut protein	ELISA	n/s (0.5)		35 min	Hazelnut Residue ELISA (ELISA Systems)
Molluscs	n/s	LFA	5 (only qual.)[a]		<10 min	RAPID 3-D Shellfish test kit (Tepnel)

n/s, not specified

LOD/LOQ, limit of detection/quantification in mg kg^{-1} (ppm) of allergenic food/target in food sample (corresponding to product name), in cases where no LOQ were reported, the lowest concentration level of the control samples supplied for quantification is given

LFD, lateral flow deviceAOAC-RI, Association of Analytical Chemist-Research Institute, validation according to the Performance Tested Method Program

BVL, German Federal Office of Consumer Protection and Food Safety, validation according to the official collection of test methods pursuant to §35 Foods and Other Commodities Act (LMBG) [30]

JRC-IRMM, Interlaboratory study organised by the European Commission Joint Research Centre - Institute for Reference Materials and Measurements [31]

[a] Matrix-dependent

[b] ppm skim milk

[c] ppm tropomyosin

[d] ppm total milk

[e] ppm non-fat dry milk

[f] ppm gliadin/gluten

chickens accumulate antibodies in their eggs, and immunisation is usually better tolerated compared with mammals, the use of antibodies derived from egg yolk is an inexpensive, convenient, and animal-friendly alternative [8–13]. The detection step involves the binding of an enzyme-labelled antibody which subsequently reacts with a specific substrate forming a coloured product. The concentration of the antigen–antibody complex can be determined by measuring the absorbance of the coloured product.

Practically all ELISA formats have found their applications in food analysis. In general, two formats can be distinguished depending on whether the detection principle is based on sandwich or competitive assays [14]. The so-called sandwich ELISA (s-ELISA) involves a capture antibody specific to the protein of interest (allergen or food marker) which is immobilized on a solid phase (e.g. microtiter plate or multiple well strips). The analytes in the sample are captured by the immobilized antibody and detected by a second analyte-specific antibody, which is enzyme-labelled and binds to the protein of interest by forming a "sandwich". If no second enzyme-labelled analyte-specific antibody exists, it is also possible to use the indirect detection procedure by also using a labelled species-specific antibody, with the "disadvantage" that both analyte-specific antibodies must be derived from different species. The absorption of the coloured product formed after adding the substrate is directly proportional to the analyte concentration (Fig. 1). Because the antigen has to offer more than one epitope in this format, s-ELISAs are only applicable to large molecules such as proteins. Thus, the sandwich assay is the most common format for allergen detection in food.

The competitive format (c-ELISA) is applicable also to the detection of small analytes. There are more possibilities to run a competitive assay. On the one hand the analyte (antigen) is directly bound to the solid phase. Sera containing the specific enzyme-labelled antibody together with the appropriately diluted sample extract are pre-incubated before addition to the antigen-coated wells. Again if no labelled antibody is available, detection can be performed indirectly. The assay consists of bound analyte, sample in solution with analyte-specific antibody, and another additional incubation step has to follow with a labelled species-specific second antibody. On the other hand the antibody can be bound to the surface and the sample analyte competes with a tracer (= enzyme-labelled

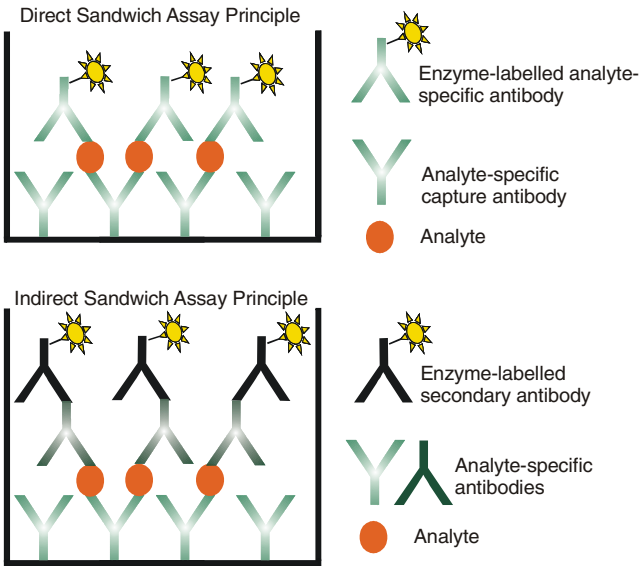

Fig. 1 Sandwich ELISA with direct detection by means of an enzyme-labelled analyte-specific antibody and the indirect sandwich assay with an enzyme-labelled species-specific secondary antibody for detection

analyte). In all three cases the antigen concentration present in the sample is indirectly proportional to the absorbance of the coloured substrate product (Fig. 2).

Rapid ELISA test kits, that produce qualitative and/or semi-quantitative results within 30 and 60 min have been developed and are commercially available for gluten, milk, soybean, peanut, hazelnut, almond, egg, and crustaceans (Table 1). With most of these ELISA kits, quantification is possible in conjunction with a microwell reader.

Lateral-flow assays (LFA) and dipstick tests

LFAs and dipstick tests for allergen detection in food are simplified versions of ELISAs with a membrane strip (usually poly(vinylidene difluoride), Nylon, or nitrocellulose), on which immunoreactants such as antibodies or antigens are applied. LFAs and dipstick tests are inexpensive, rapid, and portable, and do not require technical skill to perform. Both formats enable fast and cost-effective screening of foods because no specific instrumentation, for example microplate reader and washer, is necessary.

The technological basis for the development of immunochemical lateral-flow assays (LFA), also called strip tests, was founded in 1980, when Leuvering et al. [15] reported the use of colloidal particles as labels for immunoassays. The first, and one of the most popular, applications for this test format was use of human chorionic gonadotropin (HCG) for the detection of pregnancy [16, 17]. In 1997, Mills et al. [18] developed one of the first dipstick immunoassays used in food allergen analysis for detection of a peanut allergen (conarachin) in food. During the last decade, academic and industrial research has focused on the development of rapid allergen tests based on LFAs [19, 20] and dipstick tests [21, 22]. This has led to an explosion of rapid allergen test kits since 2003, when only two immunochromatographic allergen tests were commercially available [3]. Currently there are at least fourteen LFAs and dipstick test kits on the market, which enable the detection of the following food allergens with LODs between 1 and 25 mg allergen (allergenic food) per kg foodstuff: nuts (almond, hazelnut), crustaceans (shellfish), gluten (gliadin), peanut, milk (casein), soybean, and egg (Table 1).

LFAs are immunochromatographic tests with a mobile phase that moves the immunoreactants and/or the sample along a test strip. This type of assay satisfies the increasing demand for techniques for field and home use because they are portable, easy to operate, and rapidly produce reliable results within 3–15 min without expensive laboratory equipment and reagents. Although quantification is technically possible by using special strip test readers, commercially available LFAs are usually visually interpreted and give only qualitative or semiquantitative results, i.e. is an allergen present (yes or no) or is the allergen concentration

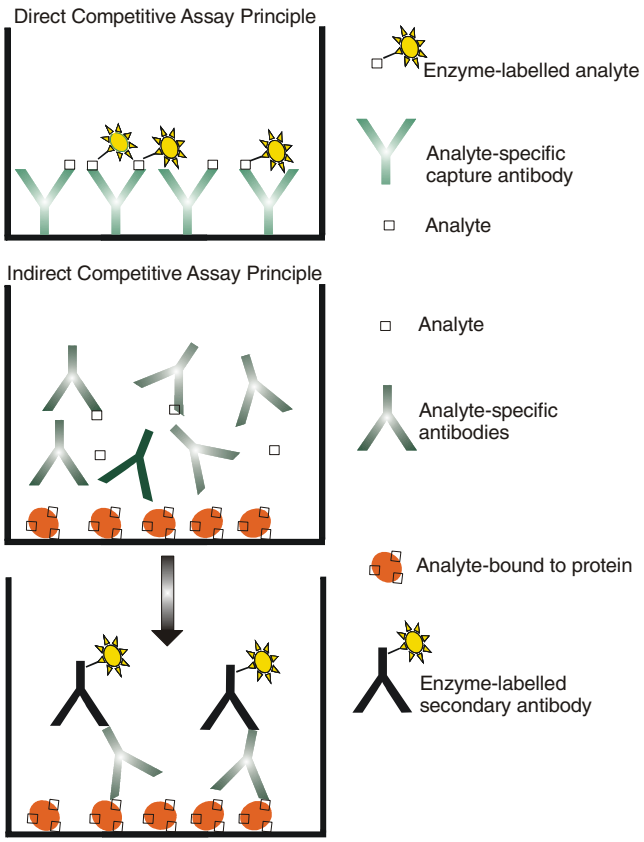

Fig. 2 Two examples of the competitive assay with direct detection by means of an enzyme-labelled tracer and with indirect detection by means of an enzyme-labelled secondary species-specific antibody

high or low. LFAs are commercially available for gliadin, egg, milk, peanut, almond, hazelnut, crustaceans, and molluscs (Table 1).

Similar to ELISA, two formats can be distinguished depending on whether the detection principle is based on sandwich or competitive format [14]. For sandwich assays, a binding agent specific for the analyte (usually, but not necessarily, an antibody) is immobilised on the membrane. The detection reagent, typically an antibody (either enzyme-labelled, or coupled to latex or colloidal metal) is deposited (but remains unbound) in the conjugate pad. When liquid sample (e.g. food extracts or liquid foods directly) is added to the conjugate pad, the detection reagent is solubilised and begins to move with the sample flow front up the membrane strip. The analyte present in the sample is bound by the antibody coupled to the detection reagent. As the sample passes over the zone to which the capture reagent has been immobilised, the analyte–detection reagent complex is trapped. Here, colour development is proportional to the amount of analyte present in the sample, which is only applicable for analytes with more than one epitope (high-molecular-mass analytes) (Fig. 3).

In competitive immunoassay procedures, the detection reagent can be the analyte bound to a protein or an antibody of the analyte bound to latex or a colloidal metal. As the analyte-containing sample and the detection reagent pass over the zone to which the capture reagent (antibody or analyte–protein conjugate) has been immobilised, some of the analyte and some of the detection reagent are bound and trapped. The more analyte present in the sample, the more effectively it will be able to compete with the immobilised analyte on the membrane for binding to the limited amount of antibodies in the detection reagent. Hence an increase in the amount of analyte in such competitive immunoassays will result in a decrease in signal in the read-out zone.

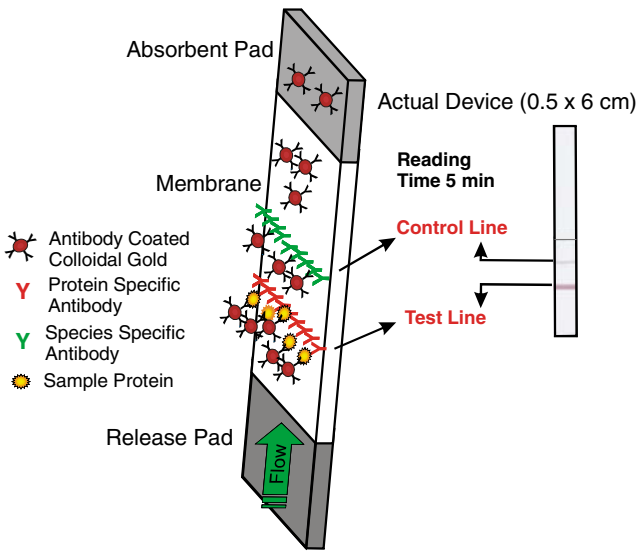

Fig. 3 Example of a lateral-flow assay based on the sandwich format

The principle of dipstick tests is similar to that of LFAs but without a mobile phase moving up the test strip. A capture antibody is immobilized on the membrane which is located at the tip of the test strip. After contact with a liquid sample, the membrane is incubated before applying a solution containing the detection antibodies. The detection antibodies are usually labelled with an enzyme which subsequently reacts with a substrate to form a coloured product. In such "sandwich" assays, the absorbance of the coloured product is directly proportional to the concentration of the analyte present in the sample. Dipsticks applying competitive ELISA as detection principle have also been reported [23–25].

The analysis time for such dipstick tests ranges from 10 min up to 3 h and depends on the number of incubation cycles involved and the time needed for all incubations. Similar to LFAs, dipstick tests provide qualitative or semi-quantitative results.

As far as we are aware, there is currently only one, actually modified, dipstick assay commercially available, which is marketed as a flow-through test for semi-quantitative determination of gluten (Hallmark). This test works similar to a dipstick assay, but with the difference that sample and reactants are dropped on to a reaction field instead of dipping a coated membrane into a solution.

Microarrays

The development of microarrays was driven by the growing demand for high-throughput, multiplexed protein analyses in the microliter to nanoliter range, and miniaturization is becoming a key element enabling maximization of the biological information gained from small, precious sample volumes [26, 27]. Microarrays contain specific (bio) molecular recognition elements (e.g. antibodies specific to a certain allergen or food marker protein) that are immobilized by microprinting or microstructuring processes and form a patterned surface. A 2D read-out can be achieved by various techniques depending on the analytical principle used for the microarray, and data analysis is achieved with software for image processing. ELISA technology or other immunoassays can be implemented by using flow-through microarray platforms that contain additionally integrated instrumentation for such a multiplexed analytical application, similar to that of a flow-injection analysis (FIA) system. Flow-through microarray platforms consist of a fluidic system for sample introduction, a reagent supply, a flow cell, a microarray on a substrate, and a detection system.

An automated and portable array biosensor developed by the Naval Research Laboratories (NRL) is capable of multiple target analysis and parallel sampling that also enables controls and standards to be analysed in parallel with unknowns [28]. The system is extremely versatile and existing immunoassay techniques can be easily implemented. Commercial versions of the portable NRL array biosensor are available (CT-ABS, Constellation Technologies; UltraRapid, Hanson Technologies), the latter also with food allergen applications.

Lateral-flow assays can also be implemented in microfluidic platforms that enable the miniaturization, integration, and automation of biochemical assays. Such microfluidic platforms can be combined with microarrays within a well defined and consistent fabrication technology to implement application-specific biochemical assays in an easy, flexible, and ideally monolithic way [29]. Miniaturized immunoassays with spotted antibodies as capture agents have been applied to the simultaneous screening of allergen-specific IgE in human serum [30]. Although only a very limited number of applications in the field of food allergen analysis have been published so far, such microarrays promise to be a powerful tool by enabling simultaneous detection of various allergens in food samples in a single step.

Biosensors

One of the emerging technologies in the past decade has been the immunochemical biosensor which enables measurement a of specific molecular interaction in real-time. Target molecules (e.g. proteins or single-stranded DNA fragments) are immobilized on a sensor chip surface and the binding activity between one or more molecules can be measured quantitatively. Immunochemical biosensors generally consist of three components: a biological receptor of appropriate specificity for the analyte, a transducer to convert the recognition event into a suitable signal, and a signal-processing system that detects, processes, and displays the analytical data. Detection and quantification is achieved by various physicochemical techniques, for example surface plasmon resonance (SPR), resonance-enhanced absorption (REA), or fluorescence evanescent-wave sensors, to name a few. In recent years, biosensors have also found increasing attention in food analysis and food allergen detection.

SPR immunoassay sensors, especially, have been frequently applied [31–34]. The increasing spread of this technology is because of the commercial availability of special SPR platforms and kits (e.g., Biacore Q, GE Healthcare and Spreeta, Texas Instruments). SPR biosensors monitor the interaction of a molecule with a prepared sensor surface in the presence of a target molecule in solution (solution inhibition) or excess analyte (surface competition). The measurable output is either a resonance angle or refractive index value; thus, there is no more need to label molecules with fluorescent or radioactive tags. The Spreeta system is an experimental SPR evaluation kit which enables time-efficient and low-cost development of immunoassay biosensors. Its feasibility for food allergen analysis has been shown with the development of a peanut biosensor [31]. The Biacore Q system has been especially developed for food analysis applications and commercial kits are available for drug residue and vitamin analysis in food. A Biacore Q optical biosensor has been used to develop both direct and sandwich immunoassays for detection of proteins from milk, egg, hazelnut, peanut, shellfish, and sesame in food samples [32]. Furthermore, a localized surface plasmon resonance (LSPR) immunosensor based on a gold-capped nanoparticle substrate has been developed for detection of casein in milk [33]. The LODs achieved with such biosensors is comparable with those of traditional ELISAs and are in the range 1 to 10 mg kg^{-1}.

Optical biosensors based on resonance-enhanced absorption (REA) have been developed for milk allergens (β-lactoglobulin) [35] and egg-white allergens (ovalbumin and ovomucoid) [36]. REA, however, needs labelled detection reagents in contrast with label-free SPR technology. To achieve a strong REA signal, the detection antibodies are labelled with monodisperse colloidal gold clusters (or gold nanoparticles). The REA effect makes use of the optical near-field phenomenon and is observed when noble metal nanoclusters are deposited at a nanometric distances from the highly reflective mirror of an interferometric set-up.

Method validation

The market for commercial allergen test kits has rapidly developed in the past five years; however data on method validation is still poor. To ensure the quality (reliability and accuracy) of analytical data produced by these test kits, it is of utmost importance to perform validations or to participate in interlaboratory comparisons.

The Performance Tested Methods Program of the AOAC Research Institute (RI) provides an independent third-party review of test kit performance claims. Test kits found to conform with their claims are granted Performance Tested Methods status by the AOAC RI. Performance Tested Methods status assures the test kit user that an independent assessment has been conducted and the kit performs as claimed. A large number of commercial test kits have been approved by the AOAC RI including four food-allergen tests (three peanut tests and one test for gliadin) [37].

A commercial hazelnut ELISA (R-Biopharm) has been validated for dark chocolate by the German Federal Office

of Consumer Protection and Food Safety (BVL). The BVL offers an "Official collection of test methods pursuant to §35 Foods and Other Commodities Act (LMBG)" as an online database [38].

In 2006, an interlaboratory study was organised by the European Commission Joint Research Centre—Institute for Reference Materials and Measurements (JRC-IRMM) [39]. Two commercially available dipstick tests (lateral flow devices) designed for detection of peanut residues in food matrixes were evaluated in an interlaboratory validation exercise with 18 participating laboratories worldwide. The test samples used in the study were cookies containing peanuts at seven different concentrations in the range of 0–30 mg peanuts kg^{-1} food matrix. However, some false-negative results were reported for all matrixes containing <21 mg peanuts kg^{-1} cookie, partly because the test kits were challenged beyond their cut-off limits (~5 mg kg^{-1}, depending on the food matrix). One test kit showed fewer false-negative results, but it led to some false-positive results for the blank materials. The sensitivity of the dipstick tests approaches that achieved with enzyme-linked immunosorbent assays.

Beside the official validation and harmonization approaches much effort is being applied to proving test results by individual researchers. On the one hand commercially available tests are subjected to inter-laboratory validation [40–43] or the individually developed immunoassays pass through adequate in-house validation. Another very important up-coming effort is the development of reference methods other than immunoanalytical detection methods, e.g. LC–MS^n methods, for detection of food allergens [44–47]. These reference methods are at the beginning of their development and, hopefully, it will soon be possible to compare immunoanalytical results with those obtained by use of these alternative methods.

Rapid tests for selected allergens in food products

Cereals containing gluten and products thereof

Gluten is the dough-forming protein fraction of wheat flour. The use of wheat flour and gluten in bakery products and other foodstuffs is extremely common because of their heat stability and useful effects on, e.g., texture, moisture retention and flavour. The new Codex Alimentarius of 2008 defines a maximum content of 20 ppm gluten in naturally gluten-free products and 200 ppm gluten in products rendered gluten-free [48].

Rapid ELISAs in microwell format for the quantification of gluten/gliadin with incubation times of only 30 min are commercially available (Table 1).

The common format used in these test kits is a sandwich ELISA that provides high sensitivity and specificity and enables quantitative analysis of prolamins from wheat (gliadin), rye (secalin), and barley (hordein) in raw and processed foods down to 2.5 mg kg^{-1} gliadin (which corresponds to 5 mg kg^{-1} gluten). LODs of the ELISA test kits range from 2 to 10 mg kg^{-1} gliadin.

There are also three commercial immunochromatographic tests (LFAs) and one commercial dipstick test (marketed as a flow-through test) for detection of gluten traces in food products. The times needed to obtain a result lies within 5 to 15 min. LODs down to 2.5 mg kg^{-1} gliadin (which corresponds to 5 mg kg^{-1} gluten) are reported (Table 1).

Crustaceans and products thereof

Shellfish and mussel are common foods in Europe. The major allergen of crustaceans is the abundant muscle protein tropomyosin and constitutes at least 20% of the soluble protein in shrimp. There is also strong immunological evidence that tropomyosin is a cross-reactive allergen among crustaceans and molluscs, in particular squid.

Two commercial test kits are available for the detection of soluble crustacean/tropomyosin levels (Table 1). A rapid ELISA test kit has been designed to provide a highly sensitive and convenient method for screening food samples for crustacean (tropomyosin) residues. The time required for testing an extracted sample is approximately 60 min. Quantification can be performed down to 0.05 mg kg^{-1} tropomyosin by using a microwell reader.

A lateral flow test for crustaceans and molluscs (including crab, lobster, brown shrimp, tiger prawn, langoustine, crayfish, scallop, oyster, mussel, cockle, and squid) has been marketed with a testing time of less than 10 min and LODs around 5 mg kg^{-1}. Because the LOD is matrix dependent, validation is recommended for each sample type.

Eggs and products thereof

Hen's egg is one of the most frequent causes of adverse reactions to foods in children. Egg can be present as an ingredient or as a contaminant in raw and cooked products. Allergenic proteins are found in egg white and egg yolk. The allergenic potential of the proteins present in egg yolk is only moderate. Threshold doses are the lowest doses at which severely egg allergic patients reacted and lie in the microgram and low milligram range.

Four rapid ELISA microwell kits have been marketed for qualitative and/or quantitative analysis of egg protein residues in various food matrices. The tests produce results within 30 to 35 min and enable quantification down to

1 mg kg^{-1} egg protein by using a microwell reader. The tests are characterised by high sensitivity (LODs down to 0.6 mg kg^{-1}) and high specificity. Sandwich ELISA formats are commonly used in these test kits, which usually apply polyclonal antibodies specific for one or more of the major egg-white proteins. A rapid immunochromatographic assay device which tests for the presence of egg proteins in various food matrix extracts within 10 min is also commercially available (see Table 1).

Baumgartner et al. [21] developed a dipstick assay for determination of egg proteins in food that has not yet reached the commercial market. The detection principle is based on a non-competitive ELISA format with direct spotting of the egg-white-specific capture antibody (raised in rabbit) on to a nitrocellulose membrane and detection with a peroxidase labelled antibody (also against egg white). The highly specific assay proved to be sensitive enough for detection of egg protein in the lower microgram per kilogram range and served well for obtaining qualitative information on the presence of egg protein in foods above 20 mg kg^{-1}. However, further research is needed to enable quantitative application of the developed dipstick assay. Another major aspect which must still be worked on is the detection of processed egg, which is highly dependent on the immunogen source the antibody was developed with. The ready-for-use dipsticks showed sufficient stability, which is essential for commercial application, and could be stored for 3 months at 6°C still with complete function.

A REA-based immunochip sensor has been developed as a rapid method for allergen detection in complex food matrices, and its application for detection of the egg white allergens ovalbumin and ovomucoid was evaluated [36]. A colorimetric solid-phase immunoassay was realized on a planar chip in direct and sandwich assay formats using antibodies functionalised with gold nanoparticles. In the direct assay format, a coating time with allergen of only 5 min was sufficient for accurate reproducibility and sensitivity. A LOD of 1 ng mL^{-1} has been achieved. The biosensor proved to be reproducible and selective in its performance and enables high-throughput screening.

Peanuts and products thereof

Peanut (*Arachis hypogeae*) is a very nutritious fruit among the legume family. Unfortunately peanuts belong to the group of most allergenic foods. Even consumption of few milligrams of peanut can induce allergic reactions in highly sensitised individuals. Because peanut allergy is persistent during life and treatment of this allergy is not possible, avoiding peanuts is extremely important for these patients. Peanut can be present as an ingredient or as traces in contaminated raw and cooked products.

For protection of consumers against "hidden peanut allergens" and for hygiene monitoring during food manufacture, intense efforts have been made by academic and industrial laboratories to develop rapid immunochemical methods.

The semi-quantitative sandwich-ELISAs in dipstick format developed by Stephan et al. [22] for detection of peanut traces in food extracts, however, involves several incubation steps that result in a total testing time of 75 min. Peanut residues as low as 1 mg kg^{-1} of peanut protein in food could be detected with this assay.

A competitive lateral-flow assay for the detection of the major peanut allergen Ara h1 has been developed on the basis of a direct competitive immunoassay using anti-Ara h1 polyclonal antibodies as capture reagents and Ara h1-tagged liposomes as detection reagents [19]. Its application was tested in chocolate and achieved results within 30 min and an LOD of 158 mg kg^{-1} of peanut in food sample [20].

Various rapid immunochemical test kits for detection and quantification of peanut residues in foodstuffs are already commercially available (Table 1). Four rapid ELISAs in microwell format currently on the market produce qualitative and quantitative results within 30 to 35 min. Sandwich ELISA again is the format of choice, because of its high specificity and sensitivity. LODs between 0.15 and 5 mg kg^{-1} have been reported. Two of the four rapid ELISAs have been approved by the Performance Tested Method Program of the AOAC Research Institute (RI) [37].

Three commercially available lateral-flow assays based on ELISA technology enable checking of the presence of peanut residues in various foodstuffs in 10 min or less with LODs between 1 to 5 mg kg^{-1}.

Soybeans and products thereof

For a long time, the allergenicity of soybeans has been underestimated. Soy is a common dietary protein and often introduced into the diet from an early age. Soy has been used as a standard milk formula in healthy children and as a substitute in children with suspected or proven cows' milk allergy. This practice, however, is now discouraged, because soy is regarded as an important food allergen, although the incidence of allergy to soybean proteins is quite low in comparison with that to other major food allergens.

Several immunochemical assays have been developed for detection of soy allergens in food products. However, they are usually time-consuming and labour-intensive. Most of these methods lack sufficient sensitivity for monitoring of soy traces in food product below the g kg^{-1} range [3].

There are currently two rapid soy allergen test kits on the market that use a sandwich ELISA format and produce results within 30 min. The sensitivities of both assays are

satisfactory, with LODs <5 mg kg^{-1}. In combination with a microwell reader, quantitative results can be obtained. Both test kits are provided by the same manufacturer (Table 1).

Milk and products thereof

Milk is a major allergenic food, especially in infancy. Cows' milk allergy must not be confused with intolerance of lactose, which is a milk sugar and does not contain protein allergens. Lactose intolerance is a consequence of lactose maldigestion which is because of genetic intestinal lactase deficiency occurring in large sections of the general population.

Several rapid immunoassay kits have been developed that enable checking for the presence of milk allergens in various foodstuffs (Table 1). Five rapid ELISA test kits are currently on the market and these deliver qualitative or quantitative results within 30 to 45 min. All these ELISA kits exhibit high sensitivity with LODs between 0.1 and 5 mg kg^{-1}. The kits either detect selected milk allergens like caseins or β-lactoglobulin, or check for the presence of total milk proteins. In products from which whey has been significantly removed, for example cheese, the estimated level of milk material, and thus also the level of β-lactoglobulin, will be lower. In these cases, a casein test kit might be more suitable to detect milk residues.

There are also two lateral-flow assays on the market that detect casein and whey residues in food products down to 0.12 mg kg^{-1} with a testing time of less than 10 min.

The applicability of an REA biosensor with a direct immunoassay on chip has been proven for rapid detection of β-lactoglobulin in food matrices [35]. A conventional ELISA for β-lactoglobulin was converted to a cluster-linked immunosorbent assay (CLISA) by labelling the read-out antibody with monodisperse colloidal gold clusters. For generation of a strong REA signal 30 min of coating of the target protein was sufficient.

Minh et al. [33] used a localized surface plasmon resonance (LSPR) immunosensor based on a gold-capped nanoparticle substrate on which anti-casein antibodies were immobilized for detection. The casein immunosensor achieved an LOD of 10 ng mL^{-1}. With the LSPR-based biosensor no time-consuming and labour-intensive labelling of detection agents is necessary and it has several advantages, for example easy fabrication, simple handling, low-cost, and high sensitivity.

Nuts and products thereof

Although nuts belong to several different botanical families, allergologically they can be grouped together. They have similarities in allergenic properties, way of consumption, clinical picture provoked, and protein pattern. Together with peanuts, nuts (particularly Brazil nuts and hazelnuts) are the most powerful food allergens. They are able to provoke severe or even fatal anaphylactic reactions at very low threshold doses. A few milligrams of nut proteins can provoke allergic reactions in sensitised individuals [1]. Currently, rapid immunochemical test kits are only available for the detection of almond and hazelnut. Therefore, the following discussion is restricted to these two tree nuts.

Almond

Almond (*Amygdalys communis L.*) is one of the most popular tree nuts worldwide because of its aromatic taste. Thus, almonds are widely used as ingredients in food products such as snacks, breakfast cereals, and bakery products (e.g. cookies, cakes, confectionary products, etc.). Moreover, almonds are a source of gourmet edible oils that potentially contain residual protein. The protein content in almonds is very high (approx. 25%) and 95% of these proteins are water-soluble, which makes them easily accessible. Even consumption of few milligrams of almond can induce allergic reactions in highly sensitised individuals.

Rapid ELISA test kits in microwell format are commercially available that enable detection of almond proteins in various food matrices with LODs ranging from 0.15 to 5 mg kg^{-1} (Table 1). Sandwich ELISAs with high sensitivity and selectivity are commonly used. Qualitative or quantitative results are obtained within 30–35 min. Strong cross reaction is observed to apricot stone, which is closely related to almond. A lateral-flow assay has been marketed and detects almond traces down to 1 mg kg^{-1} in less than 10 min.

Hazelnut

The prevalence of hazelnut allergy in the European population is 0.1 to 0.5%. Hazelnut allergy is often associated with allergy to pollens from trees like birch. Hazelnut is used as a food ingredient in pastry, confectionary products, and ice cream, but it is also processed to oils. Undeclared hazelnut might be present as cross-contaminant in various food products, for example cookies, muesli bars, or nut-free chocolate products.

There are already three rapid ELISA test kits commercially available that detect hazelnut traces in foods such as cereals, baked goods, ice cream, and chocolate (Table 1). In conjunction with a microwell reader, they can also produce quantitative results in 30 to 35 min. LODs achieved with these test kits lie between 0.15 and 5 mg kg^{-1} and depend on the degree of roasting, e.g. highly roasted hazelnuts are recovered with approximately 80% efficiency. One of the three commercial ELISA test kits has been validated for

dark chocolate following the official collection of test methods pursuant to §35 Foods and Other Commodities Act (LMBG) by the German Federal Office of Consumer Protection and Food Safety (BVL) [38].

Stephan et al. [22] developed a semi-quantitative sandwich-ELISA in dipstick format for detection of hazelnut traces in processed foods. The dipstick test, however, has a very long testing time (3–4 h) because of several incubation steps which take up to 40 min each. The limit of detection (LOD) is approximately 1 mg kg^{-1} of hazelnut protein in food.

The two LFAs on the market enable checking for the presence of hazelnut traces in various food products within 10 min. The two test kits have LODs of 1 and 5 mg kg^{-1}, respectively. High cross reactivity is observed for sesame and sunflower seeds.

Molluscs and products thereof

Although molluscs (gastropods, bivalves or cephalopods), are most often consumed in their natural form, they are also used as ingredients, after processing, in a number of preparations and in products such as surimi. The main allergenic protein in molluscs is tropomyosin, which is the same as that in crustaceans, and cases of cross-allergies between molluscs and crustaceans occur frequently. In 2006, molluscs were included in the list of allergens of the EU food labelling directive [6].

There is currently one commercial lateral flow test available that detects residues of molluscs and crustaceans down to 5 mg kg^{-1} with a total testing time of less than 10 min. LODs are matrix-specific and validation is recommended for each sample type.

Others

Lupin has been recently added to the EU list of allergens that must be labelled on food product in all circumstances [6]. Lupin flour is used in food as additive to wheat flour and in gluten-free bakery products and food for celiac disease patients. The frequency of cross-allergy between peanut and lupin is high. Recently, methods for detection of lupin in food products have been developed that are based on either ELISA technology or real-time PCR. One commercial kit based on real-time PCR for qualitative detection of lupin with an LOD of 10 mg kg^{-1} is already on the market (R-Biopharm). Cross-reactions with alfalfa (48%) and pea (0.001%) have been observed. However, no rapid immunochemical test methods that enable detection within 1 h have yet been reported.

Although fish, celery, mustard, and sesame seeds were added in 2003 to the list of allergenic foods that need to be labelled on food products [4, 5], there has been a lack of analytical methods for these four food allergens. Only recently, ELISAs [49–51, 57] and real-time PCR methods [52–55] have been developed for the determination of fish [57], celery [52, 55] mustard [49–52], and sesame [52–54] in foods. Commercial ELISA test kits are available for sesame (Tepnel, R-Biopharm, ElisaSystems) and mustard (ElisaSystems). Test kits based on real-time PCR can be obtained from R-Biopharm for all four food allergens; these achieve LODs of 5 to 10 mg kg^{-1}, depending on the allergen and food matrix. A surface plasmon resonance (SPR) biosensor has been developed for detection of the major fish allergen, parvalbumin. The SPR biosensor enables detection of parvalbumin within 5 min with LODs below 1 mg kg^{-1} and promises to be a rapid and powerful tool for allergen detection and quantification. However, for the other three allergenic foods no rapid immunochemical test with testing time of 60 min or less has yet been developed.

Buckwheat (*Fagopyrum esculentum*) has been recognized as a common food allergen in Asian countries. There is a growing interest in buckwheat products as a health food and as a substitute for wheat flour for gluten-allergic persons in Western countries. Buckwheat is not included in the food allergen list of the EU food labelling directive. It is however mentioned here in this context because a rapid ELISA test kit (ElisaSystems, Windsor) is commercially available that enables detection of buckwheat protein residues in various food matrices in approx. 45 min.

Conclusion

The demand for rapid and reliable analytical tools for the detection of food allergens in food products has increased since the implementation of the EU food labelling directive [4–6] into the legislation of many countries. In recent years many rapid test kits that check for the presence of food allergen residues within 60 min or less have become commercially available. In particular, numbers of fast ELISA test kits (testing times 30 to 35 min) and immunochromatographic tests (LFAs and dipsticks with testing times of 10 min or less) have significantly increased. Not only has analysis time been shortened, but the sensitivity and selectivity of the methods have also improved. LODs of less than 10 mg kg^{-1} of allergenic protein in a food sample have become standard and cross-reactivities that lead to false-positive results have been reduced. A lack of rapid analytical methods has been identified for several allergenic foods including celery, mustard, sesame seeds, fish, and lupin. All of these allergenic foods are listed in the EU directive for food allergen labelling and their presence in food products must be declared on food packaging.

Although much research effort has been applied to the development of optical biosensors [56] (with more than 1000 publications per year) and microarray techniques [26, 27] in the past decade, the application of these novel techniques to food allergen analysis is still limited. However, great potential has been recognised, especially for the development of portable optical biosensors, where experimental development kits are already available on the market (Spreeta and Biacore Q). Microarrays in combination with immunochemical analytical approaches offer the technical basis for simultaneous multiple-allergen detection and parallel analysis of multiple samples. With this technology, large numbers of samples can be analyzed within a relatively short time, which is essential in food screening and monitoring programmes.

The availability of rapid immunochemical allergen tests makes the task of monitoring foods for allergen contamination readily achievable, providing the industry with an important tool for quality control of raw materials, processes, and products. Increased testing will give food allergic consumers increased confidence in the safety of the food they eat, because food labelling will become more accurate and vague declarations on food labels such as "may contain traces of a specific allergen" may be widely avoided.

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3.2 Effectiveness of natural and synthetic blocking reagents and their application for detecting food allergens in enzyme-linked immunosorbent assays

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ORIGINAL PAPER

Effectiveness of natural and synthetic blocking reagents and their application for detecting food allergens in enzyme-linked immunosorbent assays

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Abstract Blocking is an important step before an enzyme-linked immunosorbent assay (ELISA) can be performed. It reduces non-specific binding to the microtiter plate to a minimum. For detecting food allergens by means of ELISA, the problem with protein blocking solutions is obvious. The blocker might interfere with the antibodies of the assay and leads to false positive results. Therefore, other blocking solutions are greatly needed. There are some alternatives like synthetic blockers or carbohydrates. Comparisons of these different blocking agents, namely proteins, carbohydrates, and synthetic blockers, were made at different reaction conditions. The incubation periods and temperatures were varied, as well as the pH. The best combinations were evaluated and compared, in respect of their blocking efficiency. The two best non-proteinaceous blockers, i.e. polyvinylalcohol and Ficoll, were subsequently applied to ELISA tests for the determination of α-casein and peanut. The study showed that Ficoll and PVA did as well as BSA in buffer solution. Therefore, they can be considered as alternative blocking reagents for ELISA, especially for the detection of food allergens.

Keywords ELISA · Blocking · PVA · Ficoll · Food allergens · α-casein

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Abbreviations

Abs	Absorbance
BSA	bovine serum albumin
ELISA	enzyme-linked immunosorbent assay
HRP	horse radish peroxidase
PBS	phosphate buffered saline
PEG	polyethyleneglycol
PN	peanut
PVA	polyvinylalcohol
PVP	polyvinylpyrrolidone
TMB	tetramethylbenzidine

Introduction

Enzyme-linked immunosorbent assays are a major tool for biochemists and analysts. The ELISA offers a wide range of possibilities concerning the specific and sensitive reaction mechanism of antibody and antigen interactions. In order to ensure the specificity of the assay, the material of the microtitre plate shall not react with the other components involved in the ELISA reaction. Blocking the bottom of the microtitre plate after coating is important for the sensitivity and specificity of the following reactants, which shall bind to the coated protein only and not onto the plate. Non-specific binding is detrimental for the assay; in addition, saturation of the unoccupied sites must be achieved [1]. The most effective and common blocking solutions consist of bovine serum albumin or casein, in other words, proteins in buffer solution. Hence, non-proteinaceous blocking reagents were resorted to the study.

Tween 20, often used as detergent in washing buffers, was tested for its ability in immunoblotting (nitrocellulose

membrane) and caused some artefacts, thus leading to a misinterpretation of results [2]. Therefore, it cannot be used alone as a blocking agent. Tween 20 and some other detergents were checked for their ability to influence protein coating onto polystyrene microtitre plates [3] since Tween 20 in PBS buffer was reported to prevent non-specific protein binding to polystyrene [4].

Other studies proved that the alternative synthetic blocker PVA reduced non-specific binding in enzyme-linked immunosorbent assays [5], as part of a post-coating solution and for stabilisation of the test kits itself [6, 7] as well as for serum pre-incubation of, e.g. Luminex assays [8]. The blocking capacity of PVP was demonstrated in [1] and in Western blotting by reducing the background signal without decreasing specific immunoreactivity [9]. Both PVA and PVP were used in ELISA for the detection of antibodies bound to virus-like particles. PVA reduced the amount of non-specific binding of antibodies, whereas PVP increased the sensitivity of antibody detection [10, 11]. Additionally, PEG was described as alternative blocker [1]. It was used as a block copolymer in a sandwich immunosorbent assay system with magnetic beads and turned out to be more effective than the conventional protein blocker BSA [12].

When working with food allergens, a more or less universal blocking agent would be of integral importance for assay development. Despite the efficiency of protein blockers, they are not the first choice in this case as they can possibly interfere with the analyte and the antibody. For example, defatted milk powder would not be used for the determination of any milk allergen in an ELISA. The application of BSA might be critical as well. Although, e.g. defatted milk protein and casein proved to be most effective for the blocking of the polystyrene microtitre plates [13], other possibilities must be taken into account if the allergen α-casein should be measured. Fish gelatine was described as an excellent blocker for nitrocellulose membranes for Western blot. Hence, it was also tested on polystyrene plates as protein blocker.

In respect of the specific ELISA systems, there are no general categorisations of blocking activity to refer to. The proteins have mainly been chosen after empirical testing and by convenience [13]. The alternative blockers PVA, PVP and PEG [1] were tested in our ELISA system for food allergens, in comparison with conventional protein blockers, like BSA and fish gelatine. Besides, carbohydrates, i.e. dextrans, Ficoll and trehalose, were taken into the series of tests to check their blocking efficiency and to evaluate a possible usage. In the first experiments, only the blocking agents were coated onto the plate, and their blocking efficiency was tracked by running simple standard curves on these plates. No dose-dependent signal changes were expected, which proved an efficient blocking strategy. Afterwards, the best-suited blockers were checked in two ELISA formats with different polyclonal antibodies for the determination of α-casein and peanut for their suitability.

Materials and methods

Reagents and instrumentation

High-binding 96-well microtitre plates from Greiner Bio One (Kremsmuenster, Austria) were chosen for the assay. For washing the microtitre plate, Tecan 96PW™ from Tecan Austria GmbH (Groedig, Austria) was employed. The 12-channel ELISA reader Sunrise™ from Tecan Austria GmbH was used with Magellan5 software.

The antibodies of rabbit and chicken (IgY) were prepared in house. IgY preparation was followed according to the protocol as described in [14]. The labelled antibodies anti-rabbit-IgG-HRP and anti-chicken-IgG-HRP were obtained from Sigma Aldrich (Vienna, Austria), as well as Ficoll (M_W~400 kDa), polyvinylpyrrolidone (M_W40–50 kDa), bovine serum albumin (Fraction V, ≥96%), fish gelatine (gelatine from cold water fish skin) and polyvinylalcohol (M_W13–23 kDa). D(+)-Trehalose (>99% purity) was purchased from Roth (Karlsruhe, Germany), polyethyleneglycol (M_W~20 kDa) from BioChemika Fluka AG (Buchs, Switzerland) and two different dextrans (M_W40 and 2,000 kDa) from Pharmacia Biotech (Vienna, Austria). The salts for phosphate buffer, citric acid and sulphuric acid were bought from Merck (Vienna, Austria). Potassium sorbic acid, polyoxyethylene-(20)-sorbitan monolaurate (Tween 20), sodium azide, 3,3′,5,5′ tetramethylbendizidine (TMB), hydrogen peroxide and dimethyl sulphoxide were purchased from Sigma Aldrich (Vienna, Austria). Water was purified by reverse osmosis before use. Samples used as blank matrices were cookies "Eierbiskotten" from Manner (Vienna, Austria) and soy milk "Alpro Soya" from Tirolmilch (Wörgl, Austria). The extraction buffer was obtained from an available Ridascreen test kit from R-Biopharm (Darmstadt, Germany). The cellulose acetate filters with glassfiber prefilters and a pore size of 0.45 μm were purchased from Sartorius Stedim Biotech (Aubagne Cedex, France).

Stock solutions and buffers

Coating buffer (pH 9.6) was made using 12 mM sodium carbonate, 38 mM sodium hydrogencarbonate and 0.01% sodium azide. A stock of 0.2 M PBS buffer containing 0.36 M NaCl, pH 7.5 was used for all phosphate buffer dilutions. Blocking solutions were varied in the assays: To dissolve 1% of blocking reagent, either 10 mM phosphate buffer containing 18 mM sodium chloride (pH 7.6) or coating buffer (pH 9.6) were used. Phosphate buffer (10 mM), containing 18 mM sodium chloride, was also

applied as washing buffer, adding 0.1% Tween 20. The assay buffer consisted of 50 mM phosphate buffer and 0.1% Tween 20. For the substrate solution, 12.5 mL substrate buffer pH 4.0, containing 0.2 M citric acid and 0.01% potassium salt of sorbic acid, was mixed with 2.5 μL hydrogen peroxide (30% *w*/*v*) and 100 μL TMB stock solution. TMB stock solution included 1.25% (*w*/*v*) tetramethylbenzidine dissolved in 5 mL dimethyl sulphoxide, then adding 25 mL methanol. H_2SO_4 (1 M) was used as stop solution. The extraction buffer for blank matrices was diluted 1:20 with deionised water.

Assay set-up for comparison of blocking reagents

Coating and blocking of the microtitre plate

Plates were coated with blocking solutions of 1% BSA, fish gelatine, PVA, PEG, Ficoll, dextran 40, dextran 2000, 3% PVP and 5% trehalose in coating buffer at pH 9.6 or in PBS buffer at pH 7.6. Blocking solution (300 μL) was filled in each well, and the sealed plate was incubated at 4 °C overnight, at room temperature for 2 h or at 37 °C for 2 h. Afterwards, it was washed three times with washing buffer containing Tween 20.

Assay procedure

α-Casein standards (100 μL; dilutions with assay buffer from 0 to 130 μg mL^{-1}) was filled into each cavity and shaken at room temperature for 1 h. The plate was washed again three times with washing buffer containing Tween 20. Rabbit-anti-α-casein (100 μL; diluted 1:10,000 with assay buffer) was added and shaken at room temperature for 1 h. After washing the plate three times with washing buffer containing Tween 20, 100 μL of anti-rabbit-HRP antibody (diluted 1:50,000 with assay buffer) was put into each well and shaken at room temperature for an hour. After the final procedure of triple washing with washing buffer containing Tween 20, 100 μL of substrate solution was filled into each cavity and shaken at room temperature for 30 min protected from light. Eventually, 100 μL stop solution was added, and the absorbance was measured at 450 nm with an ELISA reader.

Indirect sandwich immunoassay for α-casein and peanut

Coating of microtitre plate

Anti-α-casein antibody was coated onto the microtitre plate for the sandwich ELISA: IgY-anti-α-casein or rabbit-anti-α-casein was diluted with coating buffer up to a final concentration of 1 μg mL^{-1}. The sandwich was performed subject to the coated antibody used. The respective diluted antibody (100 μL) was coated onto the high-binding microtitre plate and incubated at 4 °C overnight. The washing step after coating was carried out three times with phosphate buffer without Tween 20.

For the peanut sandwich assay, anti-peanut antibody was coated. On the one hand, rabbit-anti-peanut was diluted with coating buffer up to a final concentration of 100 ng mL^{-1}, and on the other hand, IgY-anti-peanut antibody was diluted with coating buffer up to a final concentration of 2 μg mL^{-1}. The following coating procedure was the same as mentioned above for α-casein.

Blocking of the microtitre plate

Three hundred microlitres of 1% PVA in 10 mM PBS buffer or 1% Ficoll in coating buffer was used for each well of the microtitre plate as blocking reagent for the sandwich assay. Blocking with PVA was performed at 4 °C overnight, blocking with Ficoll at room temperature for 2 h. Subsequently, the plates were washed three times with washing buffer containing Tween 20.

Sandwich assay procedure

α-Casein standards (100 μL; dilutions with assay buffer from 0 to 200 μg mL^{-1}) were filled into each cavity and shaken at room temperature for 1 h. The plate was washed three times with washing buffer containing Tween 20. Secondary antibody (100 μL) was added and shaken at room temperature for 1 h. For IgY-anti-α-casein coating, rabbit anti-α-casein (diluted 1:10,000 with assay buffer) was applied as secondary antibody. For rabbit-anti-α-casein coating, IgY-anti-α-casein (diluted 1:1,000 with assay buffer) was employed. After the triple washing procedure with washing buffer containing Tween 20, 100 μL of anti-rabbit-HRP antibody (diluted 1:50,000 with assay buffer) or 100 μL of anti-IgY-HRP (diluted 1:30,000 with assay buffer) was put into the corresponding well and shaken at room temperature for an hour. The addition of substrate and stop solution was performed as for the blocking assays. The absorbance was measured at 450 nm with an ELISA reader. Four-parameter equations were formed to describe the standard curves.

For the peanut sandwich assay, 100 μL of peanut standard (dilutions with assay buffer from 0 to 400 μg mL^{-1}) was filled in each well. The assay was performed as described for α-casein, but for rabbit-anti-peanut coating, the secondary antibody was IgY-anti-peanut (diluted 1:1,000 with assay buffer), and for IgY-anti-peanut coating, the secondary antibody was rabbit-anti-peanut (diluted 1:10,000 with assay buffer). The enzyme-labelled antibody was either anti-IgY-HRP (diluted 1:30,000 with assay buffer) or anti-rabbit-HRP (diluted 1:50,000 with assay buffer).

Indirect competitive immunoassay for α-casein and peanut

Extraction procedure of blank matrices

Five grams solid sample (or 5 mL liquid sample) was extracted with 50 mL (or 45 mL) preheated extraction buffer at 60 °C for 15 min. Subsequently, the samples were centrifuged at 9,500 rpm for 10 min. The supernatant was filtered through cellulose acetate filters. The extracted matrices were treated like buffers and diluted in the same way to receive matrix standard curves.

Coating of the microtitre plate

For the competitive ELISA, α-casein was coated with 500 ng mL^{-1}, diluted in coating buffer. α-Casein solution (100 μL) was put into each cavity and incubated at 4 °C overnight. Washing was performed three times with phosphate buffer without Tween 20.

For the peanut assay, the procedure was the same as mentioned above. Peanut standard was coated in a final concentration of 500 ng mL^{-1}, diluted in coating buffer. This standard (100 μL) was put into each cavity and incubated at 4 °C overnight.

Blocking of the microtitre plate

Blocking and washing were performed as described for the sandwich ELISA for Ficoll and PVA. BSA (1%) was dissolved in coating buffer (pH 9.6) and used at room temperature for 2 h.

Assay procedure

α-Casein standard (75 μL; dilutions with assay buffer from 0 to 5,000 μg mL^{-1}) was filled into each cavity. IgY-anti-α-casein (25 μL; diluted 1:1 000 with assay buffer) or rabbit-anti-α-casein (diluted 1:10,000) was added immediately afterwards and shaken at room temperature for 1 h. After triple washing with washing buffer containing Tween 20, 100 μL of anti-IgY-HRP antibody (diluted 1:30,000 with assay buffer) or anti-rabbit-HRP (diluted 1:50,000 with assay buffer) was put into each well and shaken at room temperature for 1 h. The addition of substrate and stop solution was performed as for the sandwich ELISA. Four-parameter equations were formed to describe the standard curves.

For the competitive peanut assay, 75 μL of peanut standard (dilutions with assay buffer from 0 to 5,000 μg mL^{-1}) was filled into each cavity. IgY-anti-peanut antibody (25 μL; diluted 1:1,000 with assay buffer) or rabbit-anti-peanut (diluted 1:10,000 with assay buffer) was added immediately and shaken at room temperature for 1 h. The rest of the assay was performed as described for the sandwich ELISA.

Results and discussion

Comparison of different blocker efficiencies

Three main groups of blocking reagents were tested: proteins, synthetic reagents and carbohydrates. At first, the blocking reagents were used for blocking the plate without any other coating substance at three different temperatures and times (37 °C/2 h, room temperature/2 h and 4 °C/overnight). Additionally, two different buffers at pH 7.6 and pH 9.6 were applied. The standard curves that formed resulted from the application of α-casein and the corresponding polyclonal antibody, which was detected with a second HRP-labelled antibody. Two protein blockers, BSA and fish gelatine, and three synthetic blockers, PVA, PVP and PEG, were used, respectively. Furthermore, carbohydrates with high molecular weight were used to determine their blocking capacity and efficiency.

Blank values, preferably at absorbances around 0.1 or below, indicate an optimal blocking reagent. Higher values are an indication of non-specific binding of the standard on the microtitre plate, thus signalling insufficient blocking efficiency of the blocking reagents used. Table 1 lists the minimal, maximal absorbances, obtained by Magellan software5, and calculated differences in absorbances (Δabs) for all the blockers used at the different reaction conditions. Δabs values show the behaviour of the curve with regard to rising standard concentrations.

BSA and fish gelatine were expected to be very reliable protein blockers. However, in reality, even 1% BSA in coating buffer (pH 9.6), as common blocker, yielded absorbances up to 0.2, but there was no trend visible between blank and highest used standard concentration (130 ppb), which showed that BSA was very effective (Δabs<0.1, Fig. 1) in all conditions. Fish gelatine behaved a bit differently. At pH 7.6, incubated at room temperature and 4 °C, higher Δabs of 0.254 and 0.317 were detected, which corresponded to a visible rise in the standard curve. A comparison of the blocking efficiency of BSA with fish gelatine revealed that the latter could also be used as alternative protein blocking reagent at pH 9.6 at all incubation conditions; for pH 7.6, only the incubation at 37 °C for 2 h showed sufficient efficiency (Fig. 1). There was no enhancing trend evident for increasing standard concentrations. In general, 1% fish gelatine reached higher abs_{min} values than BSA, around 0.4 (Table 1), which has to be taken into account during the assay development.

Polyvinylalcohol, polyvinylpyrrolidone and polyethyleneglycol are the three synthetic blocking reagents

Table 1 Minimal and maximal absorbances are listed for various blocking reagents, tested at different conditions (measured at 450 nm)

Incubation temperature and period		37°C 2h			Room temperature 2h			4°C overnight		
	Buffer solution	abs_{min}	abs_{max}	Δabs	abs_{min}	abs_{max}	Δabs	abs_{min}	abs_{max}	Δabs
Proteins										
1% BSA	A	0.183	0.230	0.047	0.160	0.248	0.087	0.193	0.284	0.091
	B	0.182	0.207	0.025	0.158	0.182	0.024	0.239	0.201	0.038
1% fish gelatine	A	0.379	0.470	0.092	0.306	0.560	0.254	0.417	0.734	0.317
	B	0.304	0.367	0.063	0.306	0.342	0.036	0.350	0.386	0.036
Synthetic blockers										
1% PVA	A	0.104	0.787	0.682	0.133	0.491	0.358	0.067	0.240	0.173
	B	0.107	0.509	0.402	0.116	0.736	0.620	0.060	0.728	0.668
3% PVP	A	0.128	1.709	1.580	0.128	2.099	1.971	0.130	1.637	1.507
	B	0.155	1.698	1.543	0.154	1.653	1.499	0.129	1.587	1.458
1% PEG	A	0.116	1.311	1.196	0.081	1.465	1.384	0.091	1.240	1.148
	B	0.139	1.457	1.318	0.127	1.544	1.416	0.101	1.165	1.064
Carbohydrates										
1% dextran 40	A	0.134	2.266	2.132	0.157	2.604	2.447	0.133	1.665	1.532
	B	0.146	2.132	1.986	0.143	1.761	1.619	0.161	1.009	0.848
1% dextran 2000	A	0.140	1.515	1.375	0.125	1.547	1.422	0.156	0.828	0.672
	B	0.115	0.516	0.401	0.110	0.537	0.427	0.127	0.406	0.279
1% Ficoll	A	0.141	0.473	0.332	0.135	0.314	0.178	0.102	0.331	0.229
	B	0.204	0.182	0.023	0.130	0.184	0.054	0.123	0.209	0.086
5% trehalose	A	0.221	2.318	2.097	0.183	1.820	1.638	0.331	1.634	1.303
	B	0.163	2.164	2.000	0.135	1.629	1.493	0.268	1.667	1.399

Differences (Δabs) were calculated by subtraction of abs_{min} from abs_{max}. Each four-parameter curve was formed by Magellan software, based on the measurement of samples in triplicate. Abs_{min} and abs_{max} are parameters from this four-parameter curve. Buffer A=10 mM PBS buffer, pH 7.6; buffer B=carbonate buffer, pH 9.6

employed. As it can be seen from the Δabs values, summarised in Fig. 1, only 1% PVA at pH 7.6 incubated at 4 °C overnight showed sufficient blocking capacity. In prior studies, PVP was considered an alternative synthetic blocker, but neither 3% PVP in coating buffer nor in PBS buffer showed any blocking effects. Curves were obtained, ranging in the absorbance from 0.1 to 2.1. The same effect was shown for 1% polyethyleneglycol in coating and PBS buffer. The results were standard curves ranging in the absorbance from around 0.1 to 1.6. Yet, these results are not satisfactory. PVP and PEG can therefore not be recommended as alternative blocking agents. Only 1% PVA in PBS buffer incubated at 4 °C overnight achieved an absorbance maximum of 0.2. The Δabs reached only 0.173 (Fig. 1) in this case, indicating a comparable blocking capacity to BSA blocking. Hence, 1% PVA could be taken into account for the blocking in ELISA assays at pH 7.6. Higher amounts of PVA were not subject of these experiments because of the increasing insolubility at higher concentrations.

Finally, carbohydrates were tested as alternative blocking agents because of the great amount of hydroxyl groups available for the interaction with the activated surface of the microtitre plate. The same property is valid for synthetic reagents: They have nothing in common with the proteins to be detected; consequently, no interference with the food allergens is expected in the ELISA. The following carbohydrates were tested: Ficoll, dextran 40, dextran 2000 and trehalose. All carbohydrates were used as 1% solutions except for trehalose, which was a 5% solution dissolved in coating buffer or PBS buffer.

Ficoll is a polysaccharide made of saccharose cross-linked with epichlorohydrin to form a highly branched, high molecular weight and hydrophilic polysaccharide, which behaves neutrally and shows good solubility in aqueous solutions. The size of the Ficoll used was at about 400 kDa.

Dextran is a complex branched glucan synthesised by lactic acid bacteria. Different molecular weights are available. For this study, dextrans of an average size of 40 and 2,000 kDa were used. Trehalose was applied as stabilising agent for plate storage in the dry state due to its high water retention capability. As disaccharide, it contains two α-1,1-glycosidic linked glucose molecules. Trehalose was not able to cover the empty sites on the microtitre plate, as expected. Trehalose (5%) was dissolved in coating and PBS buffer, but

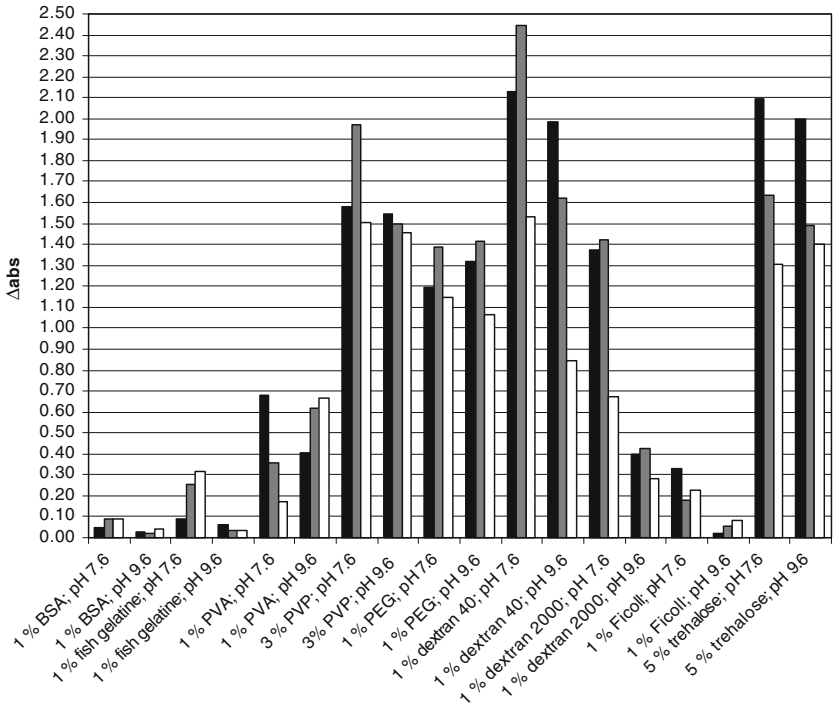

Fig. 1 Summarised data of all Δabs values (450 nm) of Table 1. Tested conditions: 37 °C 2 h (*black bars*), room temperature 2 h (*grey bars*), 4 °C overnight (*white bars*) for each blocking reagent at pH 7.6 (10 mM PBS buffer) and at pH 9.6 (coating buffer)

no blocking effects were observed, as shown in the resulting Δabs from 1.3 to 2.1 (Table 1). Standard curves were obtained within a range in absorbance from 0.2 to 2.0. Dextran 40 (1%) was dissolved in coating and PBS buffer, but the blocking results were poor. Dextran 2000 (1%) did better in both buffer solutions, but there was still a visible trend with increasing standard concentrations. Blocking with 1% Ficoll was efficient for all incubation parameters used at pH 9.6 (Δabs<0.1), but not at pH 7.6, where Δabs up to 0.33 was measured (Fig. 1).

Based on these findings, Ficoll and PVA were further investigated for their suitability as alternative "synthetic" blockers for the detection of α-casein and peanut in the sandwich and the competitive ELISA format.

Ficoll and PVA blocking for α-casein indirect sandwich and competitive ELISA format

Chicken anti-α-casein and rabbit anti-α-casein antibodies were coated and blocked with 1% Ficoll in coating buffer

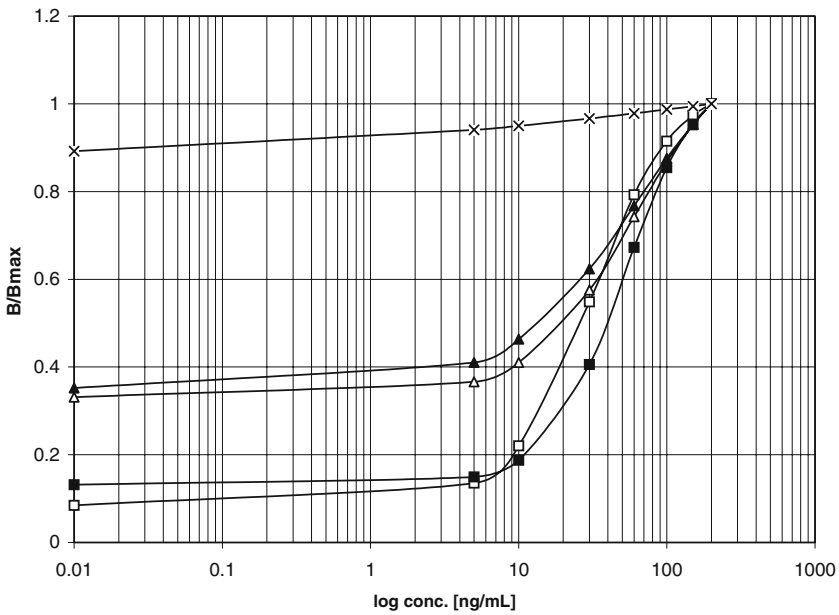

Fig. 2 α-Casein sandwich ELISA: Blocking was performed once with PVA at pH 7.6 and once with Ficoll at pH 9.6. Two sandwich formats were tested: coating 1 μg mL^{-1} rabbit-anti-α-casein with PVA blocking (*filled triangles*), coating 1 μg mL^{-1} rabbit-anti-α-casein with Ficoll blocking (*empty triangles*), coating 1 μg mL^{-1} IgY-anti-α-casein with PVA blocking (*filled squares*), coating 1 μg mL^{-1} IgY-anti-α-casein with Ficoll blocking (*empty squares*). The assays were performed without coating antibody before to check the blocking efficiency too. PVA blocking in the sandwich format with IgY-anti-α-casein coating is shown (*multiplication signs*)

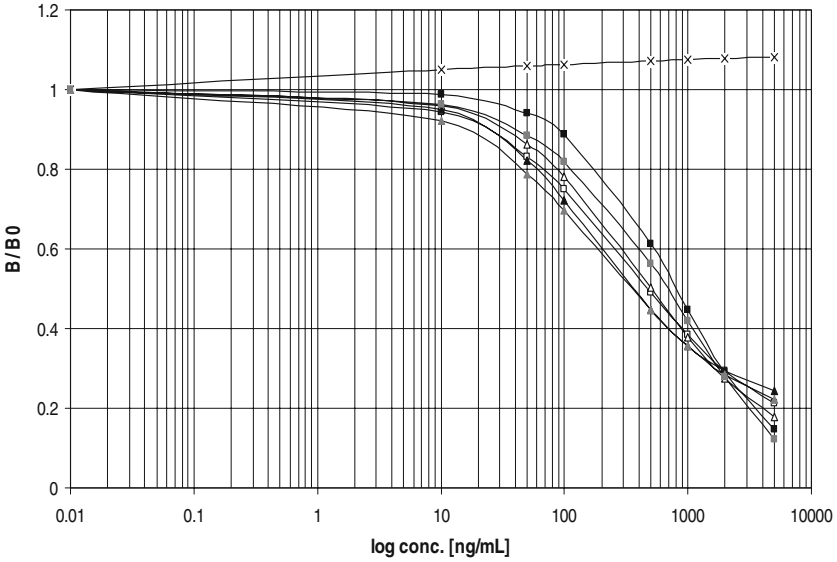

Fig. 3 α-Casein competitive ELISA: The assay was performed with rabbit-anti-α-casein and IgY-anti-α-casein. α-Casein (500 ng mL^{-1}) was coated on the microtitre plate and blocked with PVA at pH 7.6, BSA at pH 9.6 or Ficoll at pH 9.6. Rabbit-anti-α-casein with PVA blocking (*black triangles*), rabbit-anti-α-casein with BSA blocking (*grey triangles*) and rabbit-anti-α-casein with Ficoll blocking (*white triangles*) were diluted 1:10,000. IgY-anti-α-casein with PVA blocking (*black squares*), IgY-anti-α-casein with BSA blocking (*grey squares*) and IgY-anti-α-casein with Ficoll blocking (*white squares*) were diluted 1:1,000. The assays were performed without coating antibody before to check the blocking efficiency as well. PVA blocking in the sandwich format with rabbit-anti-α-casein coating is shown (*multiplication signs*)

for 2 h at room temperature or with 1% PVA in PBS buffer at 4 °C overnight. Four-parameter equations were formed with the measured absorbances by Magellan5 software. The measurements were all performed in triplicate. B/B_0 and B/B_{max} were calculated. The results for the sandwich assay are shown in Fig. 2. Each assay could be directly compared at the two different blocking conditions and they perfectly matched. There was a difference between the two formats, depending on the antibody used for coating and for detection of α-casein. The curves for rabbit-anti-α-casein coating started with higher blank values and showed a much lower increase in absorbance than the curves obtained with IgY-anti-α-casein coating.

The same assays without coated antibody showed good blocking effects in both assay formats for the corresponding blocking solutions. Hence, only one blocking curve is shown in Fig. 2. No curve in sigmoid shape was achieved, indicating the good inhibition by the blocking solution.

The same assumption was made for the competitive ELISAs shown in Fig. 3. The results were calculated in the same way as for the sandwich ELISA. The B/B_0 values were received from the four-parameter curve from Magellan5 software. Again, only one curve is shown for the blocking capacity of the two blocking reagents. Two aspects in Fig. 3 are worth considering: The standard curves for rabbit-anti-α-casein showed the same behaviour no matter if blocked with BSA, Ficoll or PVA. The blocking solutions were equally suitable. The same was shown for IgY-anti-α-casein, but the curve which was blocked with PVA showed a steeper slope than the one blocked with Ficoll. The standard curve obtained with BSA blocking can be found in between these two curves. Hence, the efficiencies of PVA and Ficoll blocking were proven.

By comparing the two types of polyclonal antibodies according to their different origin, different sensitivities can be shown with the competitive ELISA format (Fig. 3). The rabbit antibody was diluted ten times higher than the chicken antibody. However, the antibodies seemed to be equally sensitive within the appropriate dilutions in the competitive ELISA format. The curves were all very close and had a similar shape. In particular, the standard curves of both antibodies, which were blocked with Ficoll, were nearly identical.

To find out the behaviour of the synthetic and carbohydrate blocker in food matrices, one solid and one liquid sample were tested in the indirect competitive format (Figs. 4 and 5). Figure 4 shows the results of buffer and matrix standard curves blocked with Ficoll at pH 9.6. The rabbit antibody gave the same standard curves, not influenced by the different matrices used. In contrast, the chicken antibody was influenced by the cookies and the soy milk, resulting in a different shape of the curve compared to the standard curve recorded in buffer solution. Although PVA blocking had proved satisfactory results in buffer solution, Fig. 5 demonstrates high matrix effects for both antibodies used. For using PVA as blocking reagent in ELISA development, additional studies are necessary to overcome matrix effects as shown in Fig. 5.

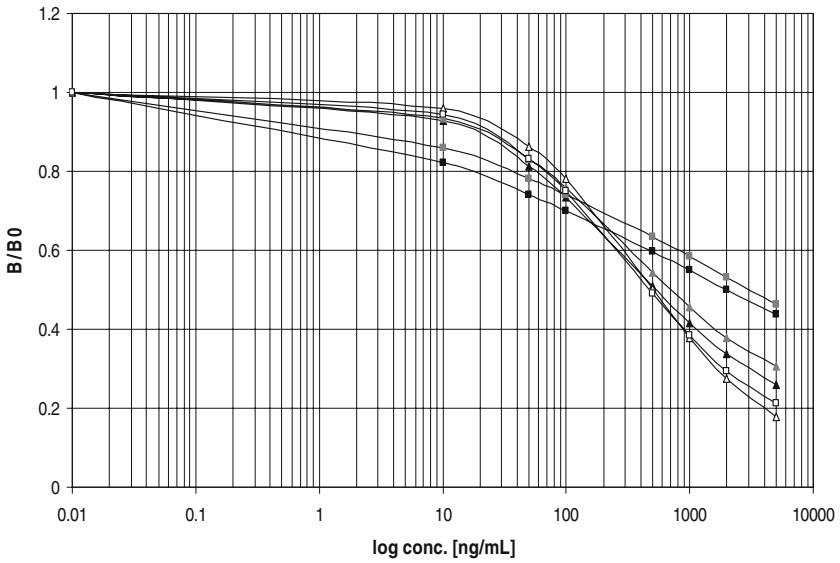

Fig. 4 α-Casein competitive ELISA: The assay was performed with rabbit-anti-α-casein and IgY-anti-α-casein. α-Casein (500 ng mL^{-1}) was coated on the microtitre plate and blocked with Ficoll at pH 9.6. Rabbit-anti-α-casein in buffer (*white triangles*), rabbit-anti-α-casein in cookie matrix (*black triangles*) and rabbit-anti-α-casein in soy milk (*grey triangles*) were diluted 1:10,000. IgY-anti-α-casein in buffer (*white squares*), IgY-anti-α-casein in cookie matrix (*black squares*) and IgY-anti-α-casein in soy milk (*grey squares*) were diluted 1:1,000

Ficoll and PVA blocking for peanut indirect sandwich and competitive ELISA format

Chicken and rabbit antibodies were coated onto the microtitre plate and blocked with 1% Ficoll in coating buffer for 2 h at room temperature and 1% PVA in 10 mM PBS buffer at 4 °C overnight. The measurements were performed in triplicate, and Magellan5 software formed a four-parameter curve out of these values, which was then applied for calculating B/B_0 and B/B_{max} values for each concentration measured. These data can be seen in Fig. 6 for the sandwich ELISA for peanut. The curves can be compared in respect of the blocking solution and of the antibodies. All curves were very close and showed the same behaviour. Both Ficoll and PVA proved to be effective blockers and yielded results that perfectly matched.

The assays were performed without coating antibodies before as well. One outcome is shown in Fig. 6 because they did not differ in behaviour. It was a straight line close to 1 for B/B_{max}, lacking non-specific binding.

The competitive ELISA assays were equally performed with chicken and rabbit antibodies and blocked with 1% Ficoll in coating buffer for 2 h at room temperature and with 1% PVA in 10 mM PBS buffer at 4 °C overnight. The calculation of the curves was done as described for the sandwich ELISA format. Figure 7 shows the results, which are comparable for both blocking solutions. Parallel standard curves were obtained with the same slope and slightly different IC_{50} values for

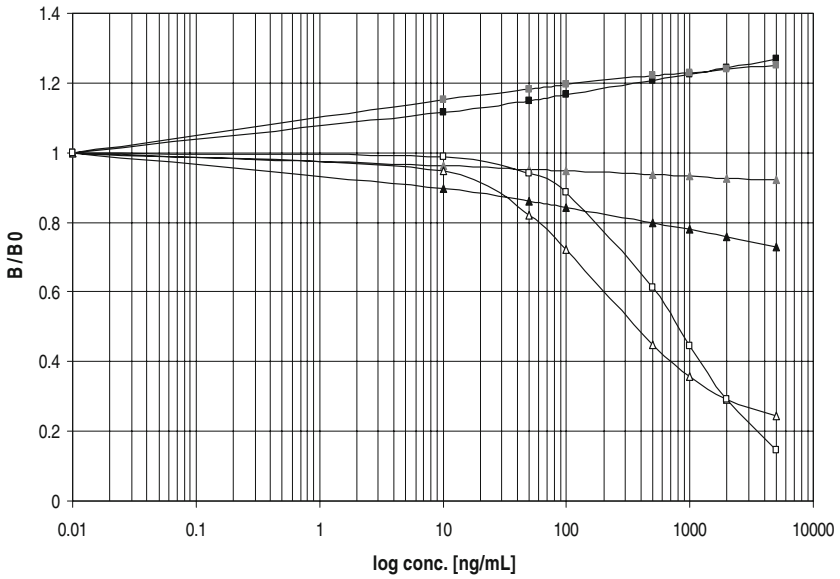

Fig. 5 α-Casein competitive ELISA: The assay was performed with rabbit-anti-α-casein and IgY-anti-α-casein. α-Casein (500 ng mL^{-1}) was coated on the microtitre plate and blocked with PVA at pH 7.6. Rabbit-anti-α-casein in buffer (*white triangles*), rabbit-anti-α-casein in cookie matrix (*black triangles*) and rabbit-anti-α-casein in soy milk (*grey triangles*) were diluted 1:10,000. IgY-anti-α-casein in buffer (*white squares*), IgY-anti-α-casein in cookie matrix (*black squares*) and IgY-anti-α-casein in soy milk (*grey squares*) were diluted 1:1,000

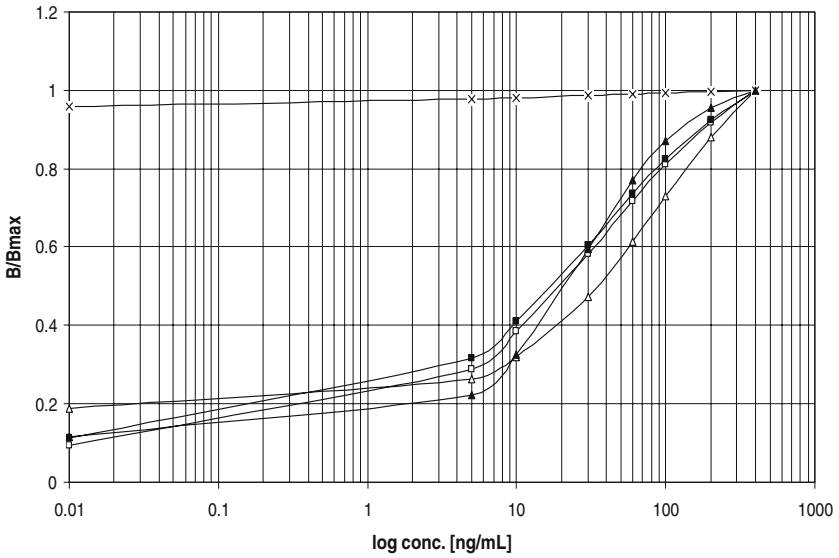

Fig. 6 Peanut sandwich ELISA: Blocking was performed once with PVA at pH 7.6 and once with Ficoll at pH 9.6. Two sandwich formats were tested: coating 0.1 μg mL^{-1} rabbit-anti-peanut with PVA blocking (*filled triangles*), coating 0.1 μg mL^{-1} rabbit-anti-peanut with Ficoll blocking (*empty triangles*), coating 2 μg mL^{-1} IgY-anti-α-peanut with PVA blocking (*filled squares*), coating 2 μg mL^{-1} IgY-anti-peanut with Ficoll blocking (*empty squares*). The assays were also performed without coating antibody before to check the blocking efficiency. PVA blocking in the sandwich format with IgY-anti-peanut coating is shown here (*multiplication signs*)

rabbit-anti-peanut antibody. For IgY-anti-peanut antibody, the IC_{50} values differ by a factor of 3, but the behaviour of the curve is similar. The blocking solutions were both effective.

The assays prepared without coating peanut did not show OD values above 0.2. Therefore, the blocking efficiency of Ficoll and PVA was established once again.

Conclusion

This study showed that non-specific binding on polystyrene microtitre plates during immunosorbent assays can be reduced by various blocking solutions. The most effective blockers remain the proteins, e.g. BSA and fish gelatine. However, for food allergens, alternatives to proteins were required. PVA, PVP and PEG were tested, representing synthetic blockers, but only PVA was capable of inhibiting non-specific binding in buffer systems. Furthermore, carbohydrates were taken into account owing to their high molecular weight and their hydroxyl groups. Dextran 40, dextran 2000, Ficoll and trehalose were considered to represent a range of different carbohydrates. Only Ficoll showed the desired blocking efficiency.

Based on these findings, two feasible blockers other than proteins remained with their specific reaction conditions: 1% Ficoll at pH 9.6 for 2 h at room temperature, 1% PVA at pH 7.6 at 4 °C overnight (only in buffer systems).

The application of Ficoll and PVA for immunosorbent assays for food allergens was shown in two examples: α-casein and peanut detection. For each food allergen,

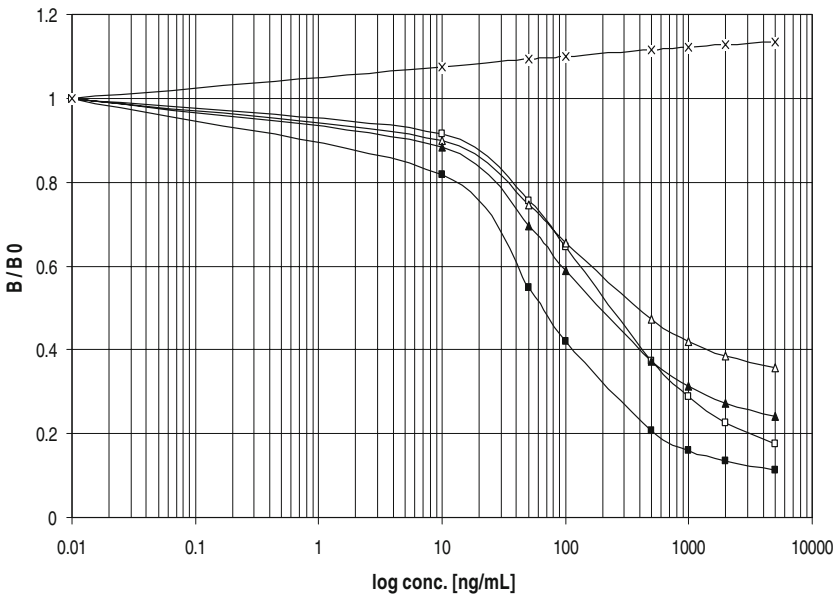

Fig. 7 Peanut competitive ELISA: The assay was performed with rabbit-anti-peanut and IgY-anti-peanut. Peanut (500 ng mL^{-1}) was coated on the microtitre plate and blocked with PVA at pH 7.6 or Ficoll at pH 9.6. Rabbit-anti-peanut with PVA blocking (*filled triangles*) and rabbit-anti-peanut with Ficoll blocking (*empty triangles*) were diluted 1:10,000. IgY-anti-peanut with PVA blocking (*filled squares*) and IgY-anti-peanut with Ficoll blocking (*filled squares*) were diluted 1:1,000. Again, the assays were run without coating antibody before to check the blocking efficiency. PVA blocking in the sandwich format with rabbit-anti-α-casein coating is shown (*multiplication signs*)

sandwich and competitive formats were performed. Both Ficoll and PVA proved suitable for the different ELISA formats in buffer systems. PVA was not capable of dealing with the matrix; in contrast, Ficoll gave comparable results in the buffer and the matrix.

Acknowledgments The authors thank the Christian Doppler Research Association and RomerLabs for funding the Christian Doppler PilotLaboratory "Rapid Test Systems for Allergenic Food Contaminants". We also thank Dr. Marcela Hermann, Institute of Medical Biochemistry, Department of Molecular Genetics, University Vienna, for the immunisation of rabbits and chicken.

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3.3 Determination of anti hazelnut antibodies specificities to different hazelnut proteins and their usability in ELISA

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Determination of anti hazelnut antibodies specificities to different hazelnut proteins and their usability in ELISA

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Abstract

Immunoanalytical assays are the method of choice for reliable detection of food allergens. The first step in their development is the production of antibodies, which can specifically recognize defined proteins. The emphasis of this study was put on producing suited preparations, and using them for the characterisation of six in-house produced monoclonal (mABs) and polyclonal (pABs) anti hazelnut antibodies, and further development of an ELISA procedure with these ABs. After extraction of ground and defatted hazelnuts with ammonium bicarbonate buffer, the extracts were dialysed against water before they were purified by a two-step FPLC method: affinity chromatography (AfC) and reversed phase chromatography. Gel electrophoresis was used for the first controlling of protein profiles before they were characterised via LC-ESI-TOF/MS. Totally, three preparations: hazelnut extract, AfC and RPC fractions have been chosen for the characterisation of antibodies. The immunological reaction of antibodies to these preparations was tested via both ELISA and western blot. The results were similar. Three mABs showed identical protein recognition patterns, which were analogue to the pattern of pAB from mouse; their IgG showed stronger binding to larger proteins. The recognition pattern of pABs from rabbit and chicken were similar to each other, too. All of pABs could recognize a protein at about 58 kDa in all of preparations that match to MW of Cor a 9. This protein could be used as marker for the detection of hazelnut ingredients in food samples. Using one of mAB and one of pAB, an indirect sandwich ELISA for detection of hazelnut in food were developed.

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Keywords Hazelnut allergens. ELISA. Antibody production. Immunoblot. Chromatographic purification. LC/ESI-TOF-MS

Abbreviations
AB/ ABs/ mAB/ pAB: Antibody/ Antibodies/ monoclonal antibody/ polyclonal antibody;
AfC: Affinity Chromatography;
ELISA: Enzyme-Linked Immunosorbent Assay;
FPLC: Fast Protein Liquid Chromatography;
HN: Hazelnut;
HRP: Horseradish Peroxidase;
LC/ESI-TOF-MS: Liquid Chromatography/ Electro spray Ionisation-Time-Of-Flight-Mass Spectrometry;
MW: Molecular Weight;
PBS: Phosphate Buffered Saline;
RPC: Reversed Phase Chromatography

Introduction

Currently Enzyme-Linked Immunosorbent Assay (ELISA) systems and DNA-based methods are the main used methods for food allergen detection [1]. The first and most important step in the development of such immunobassed assays is the preparation and characterisation of suitable antibodies (ABs). For production of antibodies, animals have to be immunized with the antigen. Especially in the branch of allergen detection, it is always discussed which immunogens have to be used to obtain different antibodies and what can be done for characterisation of the developed antibodies. The food analyst must be aware of the class of ABs being used in an immunoassay [2]. For production of polyclonal antibodies (pABs), quite often whole soluble protein extracts are used for immunisation. Raising monoclonal antibodies (mABs) would have the benefit of having a nearly “ever-lasting” antibody source, and if specific proteins are available, they could often be used as immunogens. But sometimes because of their mono-specificity these

antibodies show reduced sensitivity compared to pABs.

Due to EC-regulation 2007/68/EC [3] hazelnut (HN) is one of the 14 foods which have to be labelled as they can elicit an allergic reaction in sensitized individuals. Hazelnut consists of up to 22% carbohydrates, 24% protein, 73% fat, 3% fibre content (cellulose and pectin), mineral and some necessary vitamins such as vitamin B_1, B_6, niacin, and α-tocopherol [4]. Especially its proteins are in discussion as they can cause pollen and food related allergy, even if they are presented in trace amounts in heated and processed foods [5]. Generally plant proteins, which can cause such allergenic reactions, limited to 27 protein families and superfamilies [6] and hazelnut allergens belong to five of them. The first major allergen, Cor a 1, is a pollen related 18 kDa allergen from Bet v 1 family and subfamily of pathogenesis-related plant proteins 10 (PR-10) [7]. The second hazelnut allergen, Cor a 2 (14 kDa) belongs to the profilin family where also other allergenic proteins in both pollen and seed are found. This allergen and also Cor a 1 are labile to gastric digestions and heat treatment [8]; therefore the food allergy elicited by them is normally restricted to mild local symptoms and oral allergy syndrome [6,9]. Three other hazelnut allergens belong to the seed protein family, which is divided to cupin and prolamin superfamilies. Cor a 8 (9 kDa) is a nonspecific lipid transfer protein from the prolamin superfamily [10], which is associated with severe allergic reactions to hazelnuts. Cor a 9 (Corylin), another major food allergen present in hazelnuts, is identified as a legumin (11S seed storage globulin protein) with 59 kDa and belong to cupin superfamily; as well Cor a 11, a glycosylated vicilin (7S seed storage globulin) with molecular weight (MW) 48 kDa [11].

From the biochemical point of view, the characterisation of antibodies would be a quite challenging mission as a lot of protein purification, characterisation [10-12] and even expression of allergenic hazelnut proteins [8] in the forefront has to be done. Additionally whole protein extracts can be used for antibody characterisation but in that case a specific determination of typical binding characteristics seems difficult.

The objective of this study was extraction, purification and characterisation of hazelnut proteins from processed hazelnuts in order to prepare suitable preparations for characterisation of in-house produced anti hazelnut antibodies, which were raised against a PBS extract of ground roasted hazelnuts. Polyclonal antibodies were raised in rabbits and chicken and the obtained sera were purified and concentrated according to their typical immunoglobulin characteristics. Screening of monoclonal antibodies after the third limiting dilution was also done with the whole hazelnut extract. Clones with the highest absorbencies were chosen in first instance. In the meantime hazelnut extract was purified according to Rigby et al. [10] with the focus on Cor a 11 with slight modifications. The preparations were checked by liquid chromatography Time-of-Flight mass spectrometry (LC/TOF-MS). These preparations were then used for characterisation of in-house produced antibodies with western blot. These antibodies were additionally characterised by ELISA according to their binding ability to several recombinant and purified hazelnut proteins as well as their ability to recognise the whole extract. After comparing the results, an indirect sandwich ELISA was developed.

Experimental

Materials, instrumentations and stock solutions

Hazelnuts (roasted) were kindly provided by MasterFoods.

The anti HN antibodies from rabbit, mouse and egg (IgY) were produced in-house. The labelled species-specific secondary antibody for western blot: Anti-Mouse-IgG-HRP, Anti-Rabbit-IgG-HRP and Anti-Chicken-IgG-HRP were obtained from Sigma-Aldrich (MO, USA) as well TiterMax and also chemicals: manganese (II) chloride tetrahydrate (≥99%), ethylenediamine-tetraacetic acid (EDTA,99%), Tween20, hydrogen peroxide (30 wt.%, semiconductor grade), 3,3',5,5'-tetramethylbenzidine (TMB, 89%), potassium salt of sorbic acid, tris[hydroxymethyl]aminomethane (Trizma® base, 99.9%), Dimethysulfoxide (DMSO), and methyl α-D-manno-pyranoside (minimum 99%). Ammonium hydrogen carbonate (≥99%), Acetone (ROTISOLV®, HPLC), methanol (ROTISOLV®, HPLC gradient grade), Ficoll, 2-(*N*-Morpholino)ethanesulfonic acid (MES, minimum 99.5%), acetonitrile (HPLC gradient grade), sodiumdodecylsulfat (SDS, ≥99%), and formic acid (≥98%) were purchased from Carl Roth GmbH (Karlsruhe, Germany). Sodium carbonate, hydrochloric acid (37%) and Isopropanol (2-Propanol) was from J.T. Baker chemicals B.V. (Deventer, Holland). Di-sodium hydrogen phosphate dehydrate, sodium chloride (GR for analysis), sodium dihydrogen phosphate monohydrate (GR for analysis), calcium chloride dehydrate (GR), citric acid,

sulphuric acid, silver nitrate (GR), sodium hydroxide, and magnesium chloride hexahydrate (GR for analysis) were from Merck (Darmstadt, Germany).
NuPAGE® 12% Bis-Tris pre-cast gels (1.0 mm × 15 well), See Blue® Plus2 prestained standard (1×), NuPAGE® LDS sample buffer (4×), Simply BlueTM SafeStain, XCell IITM Blot Module and MagicMark™ XP Western Standard were purchased from Invitrogen Corporation (CA, USA). The BCA Protein Assay Kit for the determination of protein concentration and GelCode Glycoprotein Staining Kit for identification of glycoproteins were purchased from Pierce, Rockford, USA.
The chromatographic purification of both HN-extracts and antibodies was performed by using a FPLC system (Fast Protein Liquid Chromatography) from Pharmacia, Sweden; it was equipped with Pharmacia LKB Pump P-500, Pharmacia LKB FRAC-100 (collector), Pharmacia LKB Optical Unit UV-1 (detector) and Pharmacia LKB Controller LCC-501 Plus; and controlled with FPLC director version 1.03.
20 × NuPAGE® MES SDS Running Buffer was prepared by dissolving 60.6 g Trizma® base, 97.6 g MES, 10 g SDS and 3.0 g EDTA in 500 mL water. For electrophoresis, stock solution was diluted to 1× with water just before use. 0.2 M PBS (phosphate buffered saline), pH 7.5 was prepared by dissolving of 32.22 g Na_2HPO_4, 2.62 g NaH_2PO_4 and 21.18 g NaCl in 1000 mL distilled water. If required, it was diluted with water to lower molarities.
The used water was purified by reverse osmosis or was drawn from a Milli-Q plus system from Millipore (Molsheim, France). If not stated otherwise, procedures were performed at room temperature.

Production of antibodies

Polyclonal antibodies in rabbit and chicken

The polyclonal anti hazelnut antibodies in chicken and rabbit were produced as described earlier [13, 14]. The immunogen mixture was used with a protein concentration of 1 mg mL^{-1} of HN extract.

Monoclonal antibody

The 6-8 weeks old mice (Balb/c) were immunized with 100 µL immunogen mixture (0.1 mg mL^{-1}) and 100 µL TiterMax. Five subsequent boosters were performed at 2-week intervals with 100 µL adjuvant. After 4 days the blood sample was taken and screened with indirect competitive enzyme-linked immunosorbent assay (ELISA). The last booster was done after 1 week with immunogen mixture (1 mg mL^{-1}) without adjuvant; spleen cell isolation followed 4 days afterwards. Spleen lymphocytes were fused with SP²/0-AG14 mouse myeloma cells (DSMZ, German Collection of Microorganisms and Cell Cultures) using a standard procedure. Further selection and confirmation of monoclonal antibodies were also performed by indirect competitive ELISA.
The cells were centrifuged at 400 ×*g* for 5 min. The supernatant was filtered through 0.45 µm sterile filter (stericcup-HA filter system, Millipore, MA, USA). The purification of monoclonal antibodies was performed as described earlier [13] with the affinity chromatographic pre-packed HiTrap Protein G HP columns (Amersham Biosciences, Uppsala, Sweden) and the FPLC system from Pharmacia, Sweden.

Indirect competitive ELISA

The coating buffer was 50 mM sodium carbonate buffer, pH 9.6. The blocking buffer was prepared by dissolving 1% (w/v) Ficoll in coating buffer. 0.1% (v/v) Tween 20 in 0.05 M PBS was used as assay buffer, in 0.01 M PBS as washing buffer. Substrate buffer contained 0.2 M citric acid, 0.01% (w/v) potassium salt of sorbic acid and the pH was adjusted with conc. NaOH to 4. TMB stock solution was achieved by dissolving 375 mg TMB in 5 mL dimethylsulfoxide and 25 mL methanol. The substrate solution was prepared by mixing 12.5 mL substrate buffer with 2.5 µL H_2O_2 and 100 µL of TMB stock solution.
Microtiter plates were coated with 100 µL/cavity of HN solution (0.5 µg mL^{-1} HN in coating buffer) overnight at 4°C. After washing the plate three times with washing buffer without Tween 20, it was blocked with 350 µL of blocking solution for 2 h at 37°C. The plate was washed three times with washing buffer after each of the following incubation steps, except after the addition of substrate solution. 75 µL of HN standard dilutions (0-5000 µg mL^{-1} in assay buffer) and 25 µL of samples (for example serum 1:1000-25000 diluted in assay buffer or cell culture after fusion) were filled into each cavity and the plate was shacked for 60 min at room temperature. 100 µL/cavity secondary antibody (anti-species IgG-HRP peroxidase) diluted 1:10000 or 1:20000 in assay buffer was added and shacked at room temperature for 60 min. 100 µL of substrate solution was added and the plate was incubated for 30 min in darkness and room temperature before the enzymatic reaction was stopped with 30 µL/cavity 1 M H_2SO_4 and the

absorbance was measured at 450 nm with an ELISA plate reader (Tecan, Magellan). Standard curves were created by using the four parameter logistic equation.

Developing an indirect sandwich ELISA for hazelnut detection

Coating was done with the monoclonal antibody D7 (200 µL/cavity) at a dilution of 1:1000 in coating buffer at 4°C over night. Blocking was performed with 1% skim milk powder in 0.05 M PBS (400 µL/cavity) at 37 °C for one hour. The used hazelnut standard was prepared by mixing 1 g hazelnut in 10 mL buffer and the concentration of it was determined by BCA before diluting in seven steps from 200 µg/L to 2.1 µg/L. If the measured protein content by BCA is considered a factor of 8.3 is achieved for the transformation of protein concentration into nut concentration in the matrix. As detection antibody the egg yolk antibody (IgY) (200 µL/well) was used at a dilution of 1:400. Incubation was 1 hour at room temperature. Colour development was done with the anti IgY IgG HRP (200 µL/well) used at a dilution of 1:3000 with incubation for one hour at room temperature. Substrate reaction, stop solution and read out were done as mentioned above.

Producing the hazelnut preparations for chromatographic separation

Defatting and extraction of hazelnut

Twenty grams of ground hazelnuts were mixed with 300 mL acetone cooled to -20°C for 2×5 min at 4°C with an analytical mill (Ultra-Turrax T25, Janle & Kunkel; IKA-Labortechnik). After vacuum filtration (glass microfiber MN85/70 BF, Macherey-Nagel GmbH&Co., Düren, Germany) the residue was washed twice with 10 mL cooled acetone and then acetone was evaporated overnight in a fume-hood.
Proteins from roasted hazelnut were extracted with 0.1 M NH_4HCO_3 buffer (1:20) for 2 h at 4°C, followed by centrifugation at 8500 ×*g* for 30 min using an ALLEGRA® X-22 centrifuge (Beckman Coulter, Inc., Fullerton, CA, USA) to remove the solid particles. The supernatant was vacuum filtered through both glass microfiber filters and membrane filters (prefilter RW06, 47MM and membrane filter 0.45µm HA, Millipore Corporation, Billerica, USA). Further processing of the extracts included dialysis at 4°C against Milli- Q water (Spectra/Por membrane, MWCO: 6-8000, Spectrum Laboratories, rancho Dominguez, USA). After centrifugation, the sediments were resolved in the buffer and the protein concentration of the extracts was determined using BCA Protein Assay Kit according to the manual.

Chromatographic purification of Hazelnut extracts

The HN-extracts were purified using the FPLC system. The eluent was monitored for protein by measuring the absorbance at 280 nm. Protein-containing fractions were characterised by SDS-PAGE before being pooled and concentrated by ultrafiltration using a stirred ultrafiltration cell (model: 8200 from Amicon, Inc., MA, USA) or vacuum centrifuge (Model: VR-1/120/240, Heraeus Instruments, Denmark), if necessary.

a) Affinity Chromatography: Aliquots of the HN extracts were applied to a 1.0×10 cm column packed with Con A Sepharose-4B (GE Healthcare Bio-Sciences AB, Sweden) equilibrated with 20 mM Tris/HCl pH 7.4 containing 0.5 mM NaCl, 1 mM $MnCl_2$, 1 mM $CaCl_2$, 1 mM $MgCl_2$ at a flow rate of 0.75 mL min-1. After washing the unbound proteins with the same buffer, the bound protein was eluted with 0.5 M methyl α-D mannopyranoside in eluent A using a linear gradient from 0-100 %B in 60 min, 10 min hold, back in 1min, and 8 min equilibrate.

b) Reversed Phase Chromatography: Further purification was performed by RPC. The protein-containing fractions of AfC were applied to ProRPC HR 5/10 (Pharmacia, Sweden), a prepacked macroporous reversed phase C1/C8 column. Equilibration was done with 0.1% formic acid in water at a flow rate of 0.3 mL min^{-1}. After washing with the same buffer, bound protein was eluted with 0.1% formic acid in methanol.

Characterisation of chromatographically purified preparations

The chromatographic fractions were analysed by discontinuous sodium dodecyl sulfate-polyacrylamid gel electrophoresis (SDS/PAGE) and liquid chromatography/electrospray-time-of-flight-mass spectrometry (LC/ESI-TOF-MS).

SDS-PAGE and Western Blot

SDS-PAGE was performed using the pre-cast NuPAGE® 12% Bis-Tris gel following the manufacturer's instructions. The running buffer was MES SDS. After gel electrophoresis either the proteins became visible with coomassie

(using SimplyBlue™ SafeStain according to manufacturer's recommendations) or silver nitrate staining, respectively, or western blot was performed.
For blotting the XCell II™ Blot Module was used. The proteins were transferred to a 0.45 µm nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) with the transferring conditions: 30 V, 170 mA (start), and 60 minutes. The primary antibodies were in-house produced anti hazelnut from mouse, rabbit and egg yolk. The secondary anti species-specific antibody, which recognized the primary antibody, was linked to horseradish peroxidase (HRP) and used for the colorimetric detection of protein.

LC/ESI-TOF-MS

To get usable signals for the mass spectrometric determination of proteins, after desalting the samples using PD-10 columns (Amersham Pharmacia Biotech AB, Sweden), microfiltration units (Ultrafree®-MC, Millipore, MA, USA) with 10000 and 30000 NMWL (nominal molecular weight limit) were used to concentrate these proteins in the samples, and also to remove low-molecular combinations, which could later disturb the ionisation of bigger proteins by LC/ESI-TOF-MS.
Ten microliters of the filtered samples were injected into an Agilent 1100 HPLC system (Agilent Technologies, Waldbronn, Germany). This system was equipped with a vacuum degasser, autosampler, binary pump and interfaced with an ESI-TOF mass spectrometer. The HPLC separation was carried out on an ACE 3 C4-300 column, 150×3.0 mm, 3 µm particle size (Advanced Chromatography Technologies) at 40°C and a flow rate of 0.25 mL min^{-1}. Mobile phase A was 0.5% formic acid in water, and in acetonitrile was used as mobile phase B. Elution was started with linear gradient 33-45%B (0-16 min), up to 80% B in 1 min, 3 min hold, switch back to 33% B in 1 min and equilibrated for 5 min.
An ESI-TOF mass spectrometer (micro TOF™, Bruker Daltonics, Bremen, Germany) was used to determine the masses of purified HN proteins. External calibration of ESI-TOF-MS was performed prior to starting the analysis with sodium formate solution containing 5.7 mM sodium hydroxide in isopropanol and 0.2% formic acid (1:1, v/v) by syringe injection. The instrument was operated in the mass range of 500-2500 m/z and positive ionisation mode; following MS parameters were applied: nebulizer 1.2 bar, drying gas 8.5 L min^{-1}, drying temperature 200°C. The hexapole transfer parameters were: capillary exit voltage 166.7 V, hexapole RF 350.8 Vpp, skimmer 1: 78.6 V, transfer time 81.0 µs, preplus storage 10.0 µs. The flight tube voltage was -9000 V; the reflector voltage was +1300 V.

Results and discussion

Providing the HN-preparations

Extraction and dialysis

Different buffer systems were tried for extraction of hazelnut (data not shown). Since the ammonium bicarbonate buffer (0.1 M NH_4CO_3, pH 8.0) showed the best results and extraction yield, it was chosen as normally used extraction buffer. Due to the mild isolation conditions, denaturation of proteins during extraction was minimised. The extracts were then dialysed; actually to remove excessive salts that could disturb the following electrophoresis step, and also to separate still soluble and precipitated proteins. However it has been approved, that dialysis caused separation of proteins: the larger proteins, which included the HN allergens, concentrated in the sediment (Fig 1); hence the dialysis was used as a preliminary separation step.

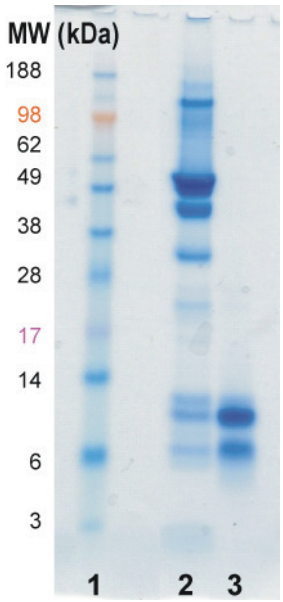

Fig 1: The protein profile of extracted HN after dialysis was checked via gel electrophoresis, coomassie blue staining; lane 1: MW marker, lane 2: proteins precipitated after dialysis; lane 3: supernatant after dialysis.

Chromatographic purification of HN extracts

Different separation technologies and mediums such as size exclusion chromatography, ion exchange chromatography, reversed phase chromatography, affinity chromatography, and

Immunoaffinity Chromatography were checked for the purification of HN extract. The fractions obtained from one single chromatographic method was not purify enough i.e. no separation of single proteins was achieved; therefore the combination of these methods was tested too. In the finally selected two-step FPLC method, the aliquot of crude HN extracts was injected into a ConA-Sepharose affinity chromatographic column. The unbound proteins were washed away and the bound proteins eluted from this column (corresponding to obtained peaks, Fig 2) applied to a ProRPC HR 5/10 reverse phase chromatography column. For a fast controlling of the efficiency of purification, and also checking the obtained protein profile, the peak-fractions were applied to SDS-PAGE before the fractions with similar protein profile were pooled.

a) Affinity chromatography: Since it is known that many plant proteins are glycosylated, the proteins from hazelnut were checked after electrophoresis directly on the SDS-PAGE gel using the GelCode Glycoprotein Staining Kit. A weak glycosylated protein in the molecular weight between 38 and 49 kDa (at about 48 kDa) was detected in all of tested HN extracts (Fig 2a, lane 7); this match to the fact that Cor a 11 is a vicillin glycoprotein with MW 48 kDa [10]. Therefore, affinity chromatography separation was performed. Glycoproteins and polysaccharides react reversibly via specific sugar residues with a group of proteins known as lectins; so Con A Sepharose-4B, a matrix to which lectin coupled as ligands, were used to isolate and separate glycoproteins. Substances bound to the lectin were resolved by using methyl α-D mannopyranoside, a competitive binding substance. Unexpectedly, the obtained fractions contained beside the 48 kDa glycosylated protein another larger protein in the MW range between 49-62 kDa (at approx. 57 kDa) (Fig 2c, lane 1-5). It is improbable that this protein correspond to an unprocessed precursor of Cor a 11. Maybe this protein was glycosylated too weakly, to be detected by the GelCode Glycoprotein Staining Kit. It is likely that this 58 kDa protein is Cor a 9, which was co-eluted with Cor a 11. Since no strict separation between these two proteins was achieved, these fractions were further purified via RPC.

b) Reversed phase chromatography: The best purified preparations were obtained with the combination of affinity and reversed phase chromatography (pre-packed ProRPC HR 5/10 column). The corresponding to peak fractions obtained from the AfC column (Fig 2b) was applied to the RPC column. The gels were visualized by silver staining. It seems that with this method the 48 kDa protein was send away and the protein in the MW between 49 and 62 kDa (at about 57 kDa) was purified; however a weak band at approx. 150 kDa became visible additionally (Fig 3a, lane 3). Interestingly this protein couldn't be detected in the AfC fraction (neither via coomassie (Fig 2c, lane 1-5) and silver staining (Fig 3a, lane 3) nor via western blot (Fig 3b-g, lane 3, 7, 11)). It is possible that this is a trimeric form of glycosylated protein that was built up through RPC conditions e.g. treatment with methanol. To take more information, these preparations were further characterised via LC-TOF/MS.

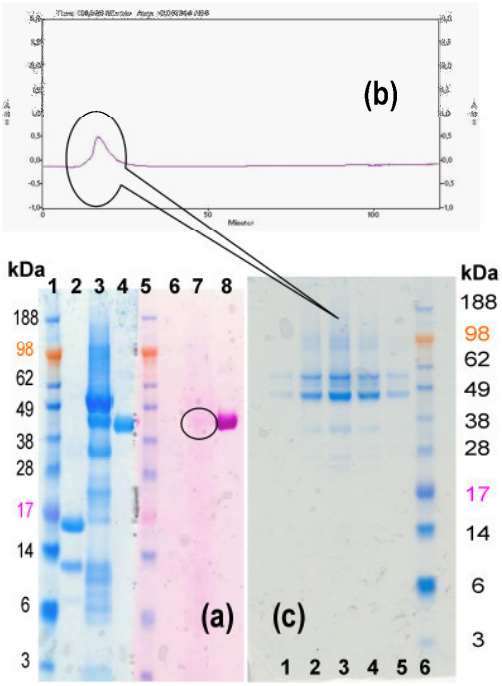

Fig 2: (a) Detection of hazelnut glycoprotein. The lanes 1-4 were stained with coomassie, lanes 5-8 with GelCode Glycoprotein Staining Kit. Lane 1 and 5: MW marker, lane 2 and 6: negative control of the kit: soybean trypsin inhibitor, lane 3 and 7: HN extract, lane 4 and 8: positive control of the kit: horseradish peroxidase. Because of glycosylated HN protein (the band between 38-49 kDa, lane 7) ConA-Sepharose separation was tried. (b) Chromatogram of AfC: the fractions correspond to this peak were applied to SDS/PAGE. (c) Protein profile of AfC peak: the gel was visualize with coomassie Staining.

Spectroscopic Characterisation of purified fractions

The mass spectrometric measurements were performed for the structural characterisation of proteins in collected fractions. The molecular weights of major HN allergens, which were calculated from their sequences, were 59 kDa for Cor a 9, 70 kDa for Cor a 10, and 45 kDa for Cor a 11; therefore the proteins in the MW range of 30 –70 kDa were sought in the fractions with a rp5-HPLC-ESI-TOF/MS.

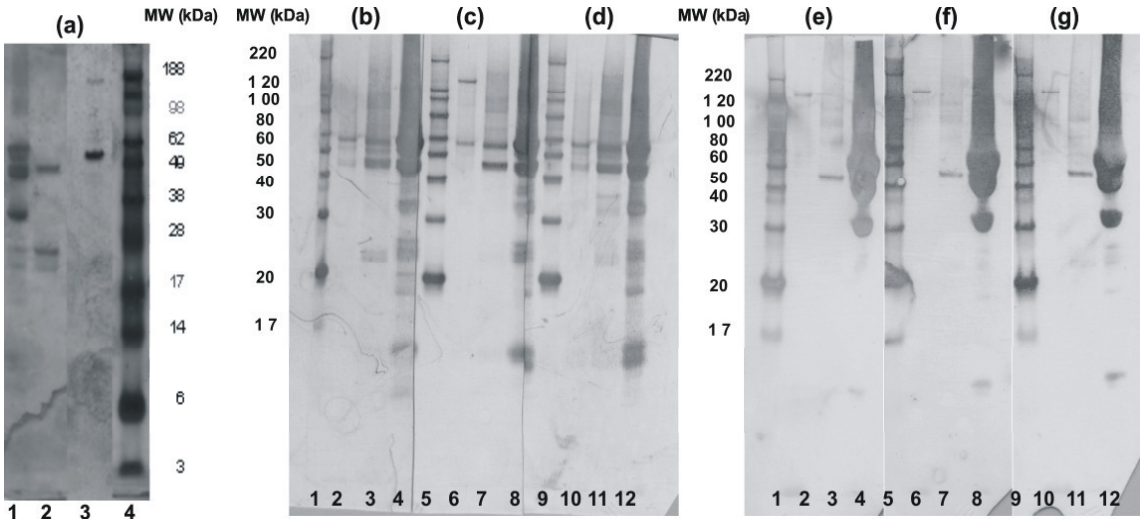

Fig 3: Protein profiles of HN preparations became visible by (a) silver staining: lane 1 HN extract, lane 2: AfC fraction, lane 3: RPC fraction, lane 4: MW marker; and by western blots using different in-house produced ABs. Polyclonal anti HN antibodies: (b) IgY, (c) IgG from mouse serum, (d) IgG from rabbit serum; and monoclonal anti HN antibodies: (e) D9, (f) D7, (g) A12. Lanes 1, 5, 9: MW marker, lanes 2, 6, 10: RPC fraction, lanes 3, 7, 11: AfC fraction, lanes 4, 8, 12: HN extract.

With mass spectrometric analysis two proteins with the molecular weights of 50 and 57 kDa at the time between 17.0-19.0 min were determined in the HN extracts (Fig. 4a) and also in the other chromatographic fractions (data not shown). The analysis of the fractions obtained from the combination of AfC and RPC showed, however, the presence of only one single protein with MW of approximately 57 kDa in the fraction (Fig. 4b); this is compatible with the smaller protein, which became visible via silver staining of AfC+RPC fractions gel (Fig. 3a, lane 3). However the another band at about 150 kDa (Fig 3a, lane 3) was probably too big to be determined via TOF-MS. Theoretically the intact proteins up to circa 140 kDa could be characterised via LC-ESI/TOF-MS, however in practice it is difficult to ionize these big molecules and therefore they can often not be detected in the mass spectrum.

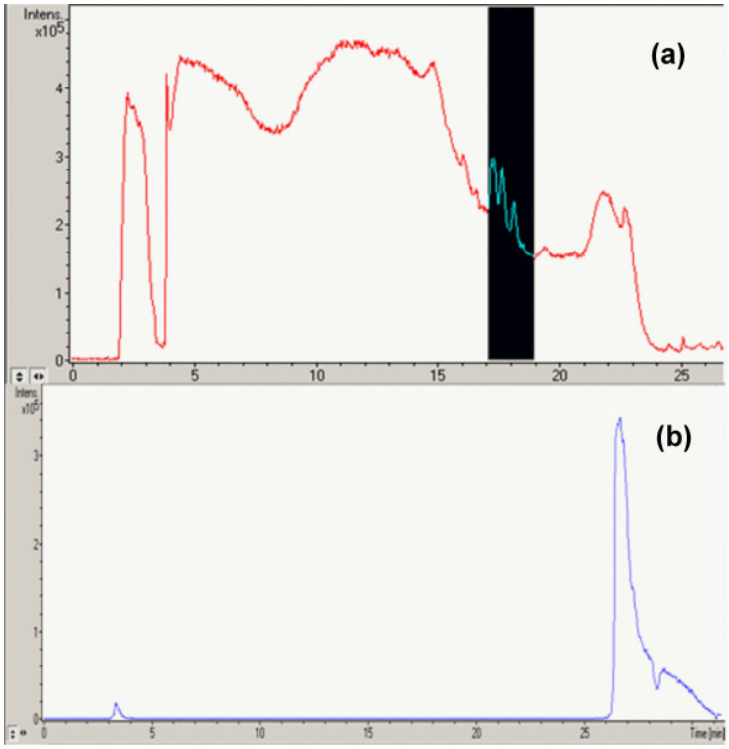

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Fig 4: Total Ion Chromatogram of ESI-TOF/MS (a) HN extracts; (b) the fraction of two-step chromatography (AfC+RPC)

Characterisation of in-house produced anti HN antibodies

The immunochemical methods, which use specific antibodies for the detection of proteins, are mostly employed, because antibodies can recognize very specific proteins in trace amounts in a complex food matrix [1]; however, the antibodies have to be very well characterized according to their properties. In this study in-house produced anti HN antibodies were characterized by both western blot and ELISA.

By Western blot

Gel electrophoresis separation of proteins (e.g. SDS-PAGE) is an important part of proteomic analyses, since in this one single step a complex mixture of proteins can be separated reproducibly and easily. But it is to be considered that the staining of protein in SDS-PAGE gels alone can hardly give accurate information about the protein identity. This can be overcome by immunoblotting procedures (such as Western blot), which provide analytical approaches for structural identification and characterisation of prepared proteins and confirm the immunoreactivity of the isolated HN proteins. In this case, the availability of appropriate antibodies is necessary to get good results for analysis. To obtain comparable and similar results, standards and reference materials are needed for characterisation of antibodies. Therefore in this work, the provided HN preparations were used.

In this context, western blots were performed to characterise in-house produced antibodies by HN preparations. Three hazelnut preparations: HN extract, HN fractions purified via AfC, and by RPC were subjected to SDS-PAGE followed by either silver staining (Fig 3a) or blotting with three polyclonal anti HN ABs: IgY (Fig 3b), mouse serum (Fig 3c), whose spleen was later used for the production of monoclonal ABs, and IgG from rabbit serum (Fig 3d), respectively three monoclonal anti HN ABs: D9 (Fig 3e), D7 (Fig 3f), and A12 (Fig 3g).

Protein recognition patterns of three in-house produced monoclonal antibodies were identical (Fig 3e-g). In HN extract (always last lane) several proteins >30 kDa were recognised. In AfC preparation (always third lane) only one band at about 48 kDa could be found (the glycosylated protein became visible via GelCode kit (Fig 2a, lane 7)). In RPC fraction (always second lane) only one protein at approximately 150 kDa could be detected, which we estimate to be a trimeric accumulation of glycolysated protein; it's compatible with the results obtained with silver staining also (the larger band in Fig 3a, lane 3). This protein could be recognized in RPC preparation by pAB anti HN from mouse too (Fig 3c, lane 6), but no binding to it was observed by pABs from rabbit (Fig 3d, lane 10) and chicken (Fig 3b, lane 2). In exchange they could find a protein at about 48 kDa (glycosylated protein); and all of three pABs could additionally recognize a band at approx. 57 kDa. Generally the recognition patterns of pABs from rabbit (Fig 3d) and chicken (Fig 3b) were similar. As indicated by recognition patterns (Fig 3b-d), all of three pABs could find multiple bands in a board mass range (6-220 kDa) in HN extract, as expected; however, the proteins recognized by AB from mouse (Fig 3c, lane 8) were fewer than the other pABs and match better to protein profile obtained via silver staining (Fig 3a, lane 1). In AfC fraction two proteins, at circa 48 (glycolysated Protein) and 57 kDa, could be recognizes by all of three pABs; pABs from rabbit and egg could find some other weaker smaller bands additionally: a protein at about 23 kDa by both of them and two other proteins at approx. 32 and 38 kDa by pAB from rabbit (Fig 3b, lane 3 and Fig 3d, lane 11). Interestingly via silver staining (Fig 3a, lane 2) only the proteins at 48 kDa (glycosylated protein) and 23 kDa became visible. The presence of the protein at about 48 kDa in AfC preparation is in accordance with the results obtained from GelCode (a band at circa 48 kDa in Fig 2a, lane 7), and literature [5,9] (48 to 50 kDa glycoprotein in HN).

In summary, IgG from mouse did not bind to low mass proteins and showed stronger binding to larger proteins. All of three mABs could recognize a strong band at approx. 150 kDa in RPC fraction and 48 kDa in AfC preparation, respectively. All of the pABs could find a protein at 57 kDa in all of preparations; the glycosylated 48 kDa protein could be detected by pABs from egg and rabbit in all preparations, but pABs from mouse could not detect it in the RPC fraction.

By ELISA

The in-house produced anti HN antibodies were also characterised by ELISA using recombinant or purified HN allergens and also HN extract (Fig 5). The results confirmed the ones obtained by western blots. As expected, pABs recognised more allergens than mAB, which recognised only Cor a 11 (48 kDa glycosylated 7S seed storage protein). The polyclonal IgY recognised only Cor a 9 and Cor a 11; the rabbit serum hat similar recognition profile, however it could additionally recognise Cor a 1 (in low concentration) and Cora a 8.

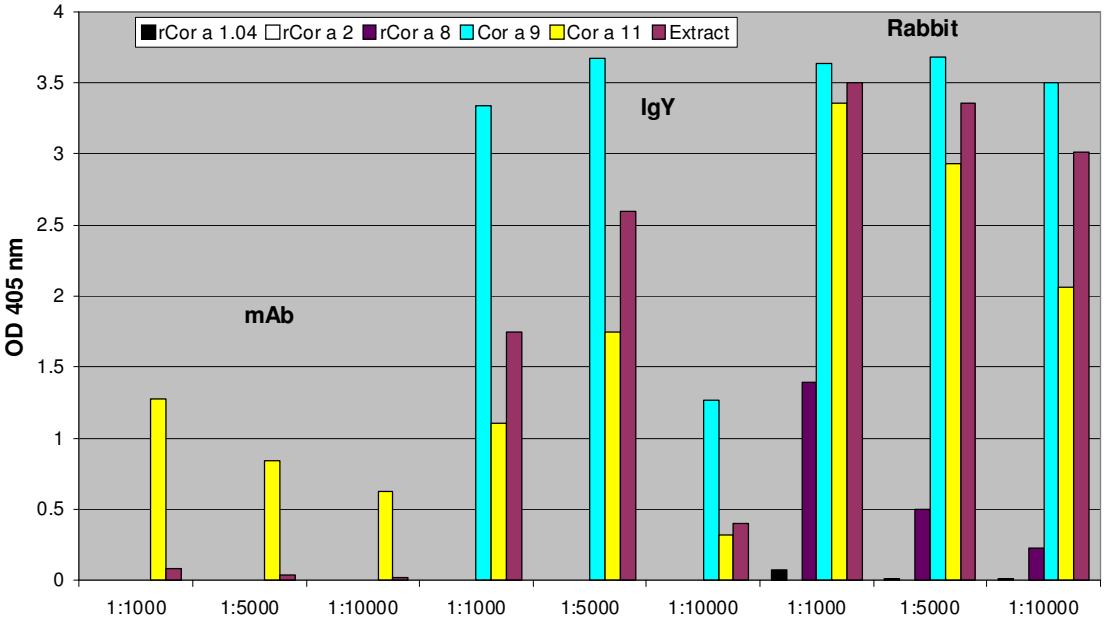

Fig 5: Characterisation of in-house produced ABs by ELISA using HN extract and also recombinant or purified HN allergens. Each ABs was tested in three dilutions.

Characteristics of developed ELISA

The monoclonal antibody D7 and the polyclonal chicken antibody were used as best suited antibody combination to set up an in-house ELISA procedure. As the monoclonal antibody showed more specific binding characteristics, less cross reactivity could be expected. Nevertheless different food stuffs were extracted and tested for cross reactivity (nuts and seeds: hazelnut, almond, macadamia, cashew nut, walnut, pistachio, chestnut, pecan, sunflower seed, pumpkin seed, poppy seed, coconut, sesame, pine nut, brazil nut; cereals: wheat, rye, corn, barley, rice, oats, linseed; legumes: soy bean, chickpea, pea, lentils, bean, peanut; fruits (dried): aranzini, candied lemon peel; other substances: lecithin, dried egg white, skim milk powder, cocoa powder, cocoa butter, yeast, vanillin, potato starch, pectin, nougat, marzipan). From them, only peanut, almond, walnut, bean, lentils, pumpkin seed, sunflower seed, cocoa powder, dried egg yolk, nougat, pine seed, pectin and yeast showed more or less cross reactivities, if the undiluted extracts were used (Table 1). Further dilution of the extracts to 100 mg/kg showed that only nougat was detected anymore, as nougat contains beside almonds also between 20 to 50% hazelnut.

The calculation of limit of detection (LOD) and limit of quantification (LOQ) could be shown that the antibodies had slight differences in LOD and LOQ, if values in buffer and matrix were compared (Table 2), where dark chocolate extracts showed the highest differencies. This is valuable information for further sample measurements because if buffer standard curves are prepared probably underestimations could be expected as this result comes from the shifted matrix standard curves as shown in Fig 6. Unfortunately the Cor a 11 purified fraction was not available in sufficient amounts to be also included as standard for the in-house ELISA.

Table 1: Cross reactivity values of various substances tested by in-house produced ELISA using D7 as capture antibody and chicken antibody as detection antibody. The extracts were measured in triplicate as undiluted and in a dilution of 1:10000 with PBS which corresponds to a concentration of 100 ppm. – not detectable

	Cross-reactivities [%]	
Commercial products	**undiluted**	**100 ppm**
Peanut	4.6	-
Almond	16.4	-
Walnut	9.3	-
Bean	0.2	-
Lentils	0.3	-
Pumpkin seed	0.6	-
Sunflower seed	0.8	-
Cocoa powder	0.3	-
Dried Egg Yolk	4	-
Nougat	100	11.8
Pine seed	1.1	-
Pectin	0.9	-
Yeast	0.4	-

Table 2: Limit of detection and Limit of quantification in standard curves diluted in assay buffer and matrix extract [mg hazelnut per kg sample]

	Assay buffer	Dark Chocolate extract	Milk Chocolate extract	Cookie extract
LOD	3.0	8.0	4.3	6.0
LOQ	9.2	23.9	12.9	17.9

Conclusion

Preparation and subsequent characterisation of suitable antibodies is the first and most important step in the development of immunoassays. For characterisation of six in-house produced monoclonal and polyclonal anti hazelnut antibodies, different HN preparations were produced. The two step FPLC system, affinity chromatography (Con A Sepharose) and reversed phase chromatography (ProRPC HR 5/10), delivered the most purified preparations. These preparations were also characterised by LC/ESI-TOF-MS. Finally three preparations: hazelnut extract, affinity and reversed phase purified fractions were chosen for the characterisation of the antibodies. The gel electrophoresis followed by western blot was the first method for the characterisation of ABs. The ABs were also characterised via ELISA according to their binding characteristics to special hazelnut proteins. The obtained results were similar. Unfortunately the obtained amount of the preparation was only sufficient for some western-blot experiments and the characterisation by LC/ESI-TOF-MS. This situation exactly demonstrates that although it is always asked for characterised standards, the needed time and effort for such natural preparations is nearly unaffordable.

Three monoclonal antibodies: A12, D7 and D9 (Fig 3e-g) showed an identical protein recognition pattern that is analogue to the pattern of polyclonal antibody from mouse, whose spleen had been used for the production of mABs; their IgG showed stronger binding to larger proteins. In AfC preparation, the mABs could find only one protein at about 48 kDa (Fig 3e-g, lane 3, 7, 11). This confirmed the presence of one glycosylated protein between 38 and 49 kDa in HN extracts (Fig 2a) and could be Cor a 11. In opposition to results obtained via TOF/MS (presence of only one protein with 57 kDa), in RPC fraction the mABs could recognize only one protein at circa 150 kDa (Fig 3 e-g, lane 2, 6, 10), however via silver staining

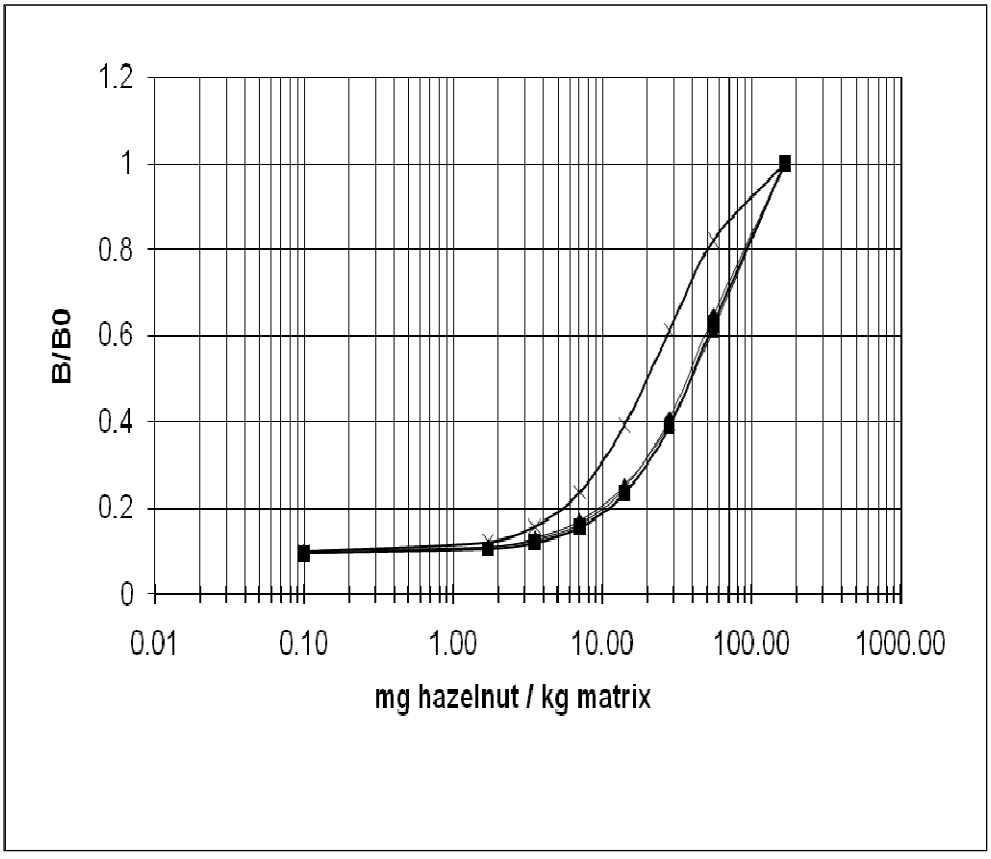

Fig 6: Assay buffer and matrix standard curves of hazelnut. -x- assay buffer, -▲- cookie, -■- milk chocolate, -♦- dark chocolate

both of them could become visible (Fig 3a, lane 3). This protein could probably be a trimeric form of the glycosylated protein, which was built because of RPC conditions (for example using the methanol for elution). Since the mABs have the suitable epitops for the glycosylated protein, they could recognize its trimeric 150 kDa protein also. This protein could not be detected by TOF/MS, because it is too big to be ionised. The other 57 kDa protein, which could be detected by TOF/MS, could not be recognized by mABs, since they do not have the appropriate epitope for it. The pABs, as expected, could detect other proteins in these preparations additionally (Fig. 5b-d, lane 3, 7, 11).

Additionally the different antibody pairs were tested for their suitability in an in-house developed ELISA and although the monoclonal antibodies showed similar results during characterization for ELISA only D7 could be used with sufficient sensitivity. In summary, the mABs are specific for 48 kDa protein; they and also pAB from mouse are the only ABs, which could recognize the trimeric protein at 150 kDa. It could be assumed that only these ABs have suitable epitops for glycosylated protein. The pABs could recognize overall proteins in HN extracts, and specially the protein at 58 kDa in all of the preparations, which could be used as marker for detection of hazelnut in food samples.

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3.4 Selection of possible marker peptides for the detection of major ruminant milk proteins in food by liquid chromatography-tandem mass spectrometry

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ORIGINAL PAPER

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Abstract The aim of this work was the determination of peptides, which can function as markers for identification of milk allergens in food samples. Emphasis was placed on two casein proteins (α- and β-casein) and two whey proteins (α-lactalbumin and β-lactoglobulin). In silico tryptic digestion provided preliminary information about the expected peptides. After tryptic digestion of four milk allergens, the analytical data obtained by combination of reversed-phase high performance liquid chromatography and quadrupole tandem mass spectrometry (LC-MS/MS) led to the identification of 26 peptides. Seven of these peptides were synthesized and used for calibration of the LC-MS/MS system. Species specificity of the selected peptides was sought by BLAST search. Among the selected peptides, only LIVTQTMK from β-lactoglobulin (*m*/*z* 467.6, charge 2+) was found to be cow milk specific and could function as a marker. Two other peptides, FFVAPF-PEVFGK from α-casein (*m*/*z* 693.3, charge 2+) and GPFPIIV from β-casein (*m*/*z* 742.5, charge 1+), occur in water buffalo milk too. The other four peptides appear in the milk of other species also and can be used as markers for ruminant species milk. Using these seven peptides, a multianalyte MS-based method was developed. For the establishment of the method, it was applied at first to different dairy samples, and then to chocolate and blank samples, and the peptides could be determined down to 1 ng/mL in food samples. At the end, spiked samples were measured, where the target peptides could be detected with a high recovery (over 50%).

Keywords Milk allergens · α-Casein · β-Casein · α-Lactalbumin · β-Lactoglobulin · Liquid chromatography-tandem mass spectrometry

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Introduction

Milk is one of the most common and widespread allergenic foods. Milk allergy is an adverse immunological reaction to milk proteins of different mammalian species. The milk of different ruminant species (e.g. cow, buffalo, sheep and goat) as well as that of humans, contains the same or closely homologous proteins which share the same structural, functional and biological properties [1].

About 3.2% of milk is protein, which is subdivided into two fractions: casein accounts for 80% of the milk protein and whey for the other 20% [2]. The caseins (α-casein 42% and β-casein 28%) are found in the micelles, giving milk its cloudy appearance [3]. The whey fraction contains two major components: β-lactoglobulin (BLG; 10%) and α-lactalbumin (ALA; 5%). Studies have shown that due to the great variability of human IgE response, no single allergen or particular structure can account for a major part of milk allergenicity, and polysensitization to several proteins is also common. All milk proteins, even proteins present at low concentrations, appear to be potential allergens; however, the caseins, BLG and ALA are major allergens (50–65% of patients), and sensitization has been demonstrated to be closely linked [1, 4–6].

It seems that even trace amounts of milk allergens can induce allergic reactions in patients and result in mild to

fatal health problems. However, available data and reports from controlled food challenges do not permit the establishment of validated threshold doses for specific allergic reactions or the determination of a level of exposure that could protect allergic consumers against a reaction to allergens present in their food [1, 7, 8]. The threshold levels vary from patient to patient, depending on the food concerned to the sensitivity of selected allergic individuals. Due to the fact that currently the only effective treatment is total avoidance of the offending food, EU regulation enforces the declaration (Directive 2007/68/EC amending Annex IIIa to Directive 2000/13/EC) of 14 food allergens, including milk and products thereof. However, the detection of allergens in food products can be very difficult because often only trace amounts are present or they are masked by the food matrix. Thus, reliable, sensitive and specific analytical methods allowing accurate identification of milk or milk components in food samples are sought. These approaches could target either the allergen itself or a marker that indicates the presence of the offending food and food component [9]. The methods for the detection of proteins in general include almost exclusively immunoanalytical methods, which use specific antibodies for the detection of target proteins, such as immunoblotting or enzyme-linked immunosorbent assay [10]. However, heating and technological food processing might lead to changes in target-protein structure, affecting final antibody-based detection. In order to overcome these drawbacks, mass-spectrometry (MS)-based methods have been developed which are not dependent on antibodies and could therefore offer a complementary tool, useful for final confirmation of the presence of an allergen in different commodities [11].

MS analysis has been applied in the last several decades in broad diverse objectives, including the determination of molecular weight for complete protein and sequencing of peptides from enzymatic mapping for structural characterization [12]. More specific analysis comprises the digestion of the sample with an enzyme such as trypsin, followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis to derive the amino acid sequences of individual peptides of the proteolytic digest (bottom-up approach) [2, 13]. The analytical throughput and efficiency of top-down proteomics are still limited for large-scale proteome analysis. Peptide-level measurements are generally more effective for protein identification than mass spectrometric analysis of intact proteins, and the accuracy of mass detection is more precise for small molecules. Also, specificity and selectivity is a major requirement, which can be increased for the whole analysis method with the choice of many target peptides (multianalyte method) [10]. Most of the publications, which deal with the detection of milk allergens, analyzed intact milk proteins [e.g. 8]. Monaci and van Hengel [7] developed a method based on LC coupled with electrospray ionization (ESI)-MS detection to identify and quantify, again, intact allergenic whey proteins in mixed fruit juices with a limit of detection down to 1 μg/mL.

On the one hand, different MS techniques were used for the analysis of milk proteins of different ruminant species [e.g. 14, 15] or for the determination of the sequences of the tryptic peptides derived thereof [e.g. 16, 17]. On the other hand, numerous allergenic IgE and IgG binding epitopes of each of the four milk allergens were investigated to determine the allergenicity of the milk allergens [1, 3–6, 16]. Despite these two facts, literature is scarce in the field of mass spectrometric detection of allergenic milk proteins at low concentrations in food products and specially the combining of two above-mentioned techniques (using peptides as marker for the identification of whole milk allergens by MS). Very recently, Monaci et al. [18, 19] used ESI-Q-TOF MS for the identification of peptides derived from casein. In the first publication [18], they detected some peptides only from caseins (α and β) at 100 and 1,000 μg/mL in fined white wine. In a technical note [19], they detected two peptides derived from α-casein as markers. As they rely on a comparison of retention times in LC-MS full scan data with compound confirmation by data-dependent MS/MS product ion scan data of standard and matrix samples, the risk of negative influence of unknown co-eluting matrix components on the signals from chosen diagnostic tryptic peptides cannot be excluded.

In this work, mass spectrometric techniques have been used for the detection of seven peptide markers arising from both casein (α and β) and whey milk proteins (α-lactalbumin and β-lactoglobulin). A multianalyte method was developed, which is capable to detect these diagnostic tryptic peptides from different milk proteins in a single analysis run. For the establishment of the method, it was applied at first to different dairy, blank and chocolate samples and then to spiked samples, wherein the target peptides could be detected with a high recovery. This study is the first step to establish a possible alternative and comparative method to immunoanalytical methods, which could be used as a reference method for the determination of milk allergens in different food samples.

Material and methods

Materials, chemicals and reagents

Milk and dairy products (parmesan, mozzarella, yogurt, sheep yogurt and whey drink) and also chocolate products were purchased from local markets. The proteins (all from bovine milk) α-casein (minimum 70% α_S-casein), β-casein (minimum 90%), α-lactalbumin (≥85%) and β-lactoglobulin (approximately 90%) and also the chemicals ammonium

acetate (MS grade) and iodoacetamide were obtained from Sigma-Aldrich (Steinheim, Germany). Ammonium hydrogen carbonate (≥99%), sodium hydrogen carbonate (≥99.5%), 1,4-dithiothreitol (≥99%), milk powder, acetonitrile (HPLC gradient grade) and formic acid (≥98%) were purchased from Carl Roth GmbH (Karlsruhe, Germany). Trypsin (sequencing grade, modified) was from Roche Applied Science (Mannheim, Germany). Urea and di-sodium hydrogen phosphate dihydrate were from Merck (Germany). Sodium hydroxide was from Fluka (Buchs, Switzerland). Water was drawn from a Milli-Q plus System from Millipore (Molsheim, France).

Target peptides Seven peptides have been chosen (one for α-lactalbumin and two for each other selected protein, which showed the highest MS signal) and ordered in lyophilized form from 'Peptide 2.0' (Chantilly, VA, USA).

If not stated otherwise, procedures were performed at room temperature.

Sample preparation (extraction, digestion and spiking)

For extraction, 0.4 g of sample was mixed with 4 mL of extraction buffer (50 mM $NaHCO_3$, pH 9.6). The mixture was heated at 60 °C for 15 min in a water bath (GFL, Burgwedel, Germany) and centrifuged for 20 min at 6,500×*g* and 4 °C using a Conical Tube Fixed-angle Rotor (C1015) and an ALLEGRA® X-22 centrifuge (Beckman Coulter, Inc., Fullerton, CA, USA). The supernatant was filtered through both prefilter RW06, 47MM and membrane filter 0.45 μm HA from Millipore (Millipore Corporation, Billerica, MA, USA) and glass microfiber MN85/70 BF from Macherey-Nagel (Macherey-Nagel GmbH & Co., Düren, Germany). The filtrate was dried completely overnight using a vacuum centrifuge, Model VR-1/120/240 (Heraeus Instruments, Denmark).

Proteolytic digestions with trypsin were carried out using a protocol for in-solution digestion [20]: In short, the extracted proteins were resuspended in a 100 mM NH_4HCO_3 buffer pH 8 containing 6 M urea. One hundred-microlitre aliquots were reduced and alkylated using 200 mM DTT and 200 mM iodoacetamide in 100 mM NH_4HCO_3. One hundred-microlitre of a trypsin solution (200 ng/μL trypsin in 100 mM NH_4HCO_3) was added to the samples and incubated at 37 °C overnight. The reaction was stopped by addition of formic acid.

For spiking, both milk and mixtures of synthesized peptides were used. For determining the effect of procedures, the samples were spiked either before or after extraction, prior to digestion. For milk spiking before extraction, different volumes of milk was added to 1 g ground peanut puffs. For milk spiking after extraction, 100 μL milk were added to 900 μL peanut puffs extracted with 50 mM $NaHCO_3$, pH 9.6. Also, for testing the matrix effect, 24 μL of standard peptide mixture with different concentrations (37, 111, 370 and 1,111 ng/mL) were added to 80 μL of a 1:100 diluted digested food sample. The volume was adjusted with 50% aqueous acetonitrile (*v*/*v*) containing 5 mM ammonium acetate to 240 μL.

High performance liquid chromatography (HPLC)

Chromatographic separation of tryptic digested samples was carried out at 25 °C on a Zorbax SB-C_{18} reversed phase column, 150×2.1 mm, 3.5 μm particle size (Agilent, Waldbronn, Germany), equipped with a C_{18} 4×3 mm i.d. security guard cartridge (Phenomenex, Torrance, CA, USA) and using an HPLC series 1100 (Agilent, Waldbronn, Germany). Injection volume was 5 μL, and the flow rate was 0.35 mL/min. Eluent A consisted of 10% aqueous acetonitrile (*v*/*v*), and eluent B was 95% aqueous acetonitrile (*v*/*v*). Both HPLC eluents contained 5 mM ammonium acetate. The following HPLC program was used: initial hold of 0.5 min, gradient from 0% to 100% eluent B within 10.5 min, 3.5 min hold, switchback of the mobile phase to eluent A within 2 min and equilibration of the column for 12.5 min.

Electrospray tandem mass spectrometry

A QTrap 4000 LC-MS/MS system (Applied Biosystems, Foster City, CA, USA) equipped with a 'Turbo V Ion Spray' ESI source was used. Screening and identification of tryptic peptides, as well as optimization of SRM parameters for profiling of selected tryptic peptides, were carried out by directly infusing of tryptic digested milk proteins (concentration, 25–50 mg/L) or of peptide standard solutions (with a concentration of 140–330 μg/L), respectively, into the mass spectrometer at a flow rate of 10 μL/min; all were dissolved in 50% aqueous acetonitrile (*v*/*v*) and 0.1% formic acid (*v*/*v*).

The ESI source was used in positive ionization mode with the following parameters: curtain gas, 69 kPa (10 psi); ion source gas 1 (sheath gas), 207 kPa (30 psi); ion source gas 2 (drying gas), 345 kPa (50 psi); source temperature, 150 °C; and ion spray voltage, +4,000 V. Collision gas was set to high. Nitrogen was used for all gases. For each SRM transition, a dwell time of 50 ms was chosen, and the pause between mass ranges was 5 ms. The optimized SRM parameters (Table 1) were used for profiling of tryptic peptides of milk allergens by LC-MS/MS.

For the use of the QTrap4000 in LC-MS/MS experiments, the ESI source was used with the following settings: gas 1, 345 kPa (50 psi); gas 2, 345 kPa (50 psi); and source temperature, 535 °C. For the other parameters, the same values were chosen as for the SRM optimizations. Chromatographic and (tandem) mass spectrometric data were evaluated using the Analyst™ software version 1.5.

MS full scans were performed in the Q1-MS mode (the first quadrupole scans, the other quadrupoles transfer the

Table 1 Parameters of the LC-MS/MS method in SRM mode for profiling of tryptic peptides of milk allergen proteins

Protein		MW (Da)	Q1 (*m/z*)	Q1 ion	Q3 (*m/z*)	Q3 ion	DP (V)	CE (V)	CXP (V)	
α-Lactalbumin	VGINYWLAHK[a]	1,199.6	601.2	$[M+2\ H]^{2+}$	284.4	y_2	81	41	14	Q
	11/131[b]		601.2		355.4	y_3	81	41	30	
	3,704/43,764[c]		601.2		654.4	y_5	81	33	8	
β-Lactoglobulin	IPAVFK[a]	673.5	338.0	$[M+2\ H]^{2+}$	282.0	b_3	41	21	14	
	1/30[b]		338.0		294.4	y_2	41	27	18	
	1,111/30,115[c]		338.0		561.6	y_5	41	19	8	Q
	LIVTQTMK[a]	932.5	467.6	$[M+2\ H]^{2+}$	227.4	b_2	46	21	14	
	4/73[b]		467.6		608.6	y_5	46	23	8	
	1,111/21,756[c]		467.6		707.6	y_6	46	21	10	Q
α-Casein	YLGYLEQLLR[a]	1,266.7	634.8	$[M+2\ H]^{2+}$	249.4	a_2	56	35	12	Q
	1/20[b]		634.8		771.8	y_6	56	33	12	
	3,704/67,128[c]		634.8		991.7	y_8	56	31	16	
	FFVAPFPEVFGK[a]	1,383.7	693.3	$[M+2\ H]^{2+}$	267.3	a_2	56	47	18	Q
	1/18[b]		693.3		676.6	y_6	56	43	10	
	3,704/61,455[c]		693.3		920.8	y_8	56	29	16	
β-Casein	GPFPIIV[a]	741.4	742.5	$[M+H]^{+}$	441.5	y_4	101	41	12	Q
	1/35[b]		742.5		512.4	b_5	101	41	16	
	3,704/117,654[c]		742.5		625.6	b_6	101	35	10	
	VLPVPQK[a]	779.5	780.6	$[M+H]^{+}$	213.2	b_2	101	61	12	
	1/34[b]		780.6		372.4	y_3	101	51	10	Q
	3,704/111,919[c]		780.6		568.6	y_5	101	45	16	

Working ranges were obtained in a calibration analysis: seven peptides dissolved in organic solvent (standard mixture, injected at eight concentration levels). The protein concentrations were calculated from the concentration of according peptide masses

LOD limit of detection, *LR* linear range, *Q1* precursor ion, *Q3* product ion, *DP* declustering potential, *CE* collision energy, *CXP* cell exit potential, *V* volts, *Q* quantifier

[a]Tryptic peptide

[b]LOD: peptide/protein (ng/ml)

[c]LR: peptide/protein (ng/ml)

ions to the detector) in the mass range 350–1,500 and 1,500–2,800 Da for a duration of 1.5 min each (corresponding to 45 accumulated scans each). Charge states of the precursor ions of intact tryptic peptides were confirmed by measurements in the enhanced resolution (ER) scan mode, hereby scanning a range of 15 Da around the precursors (*m/z* of precursor ±15 Da). In the EPI mode, the precursor ions were fragmented with a collision energy (CE) of 50±20 V collision energy spread, and the resulting product ions were scanned at a speed of 4,000 Da/s in the mass range 50 Da to *m/z* of precursor ion +20 Da, corresponding to 30 accumulated scans. Declustering potential for MS full scans and EPI was 70 V. Dwell time for each SRM transition was chosen 50 ms.

Calibration of the LC-MS/MS method in SRM mode

For the determination of the concentration of the peptides in the tryptic digested food samples, the LC-MS/MS method in SRM mode was calibrated by analyzing eight dilution levels of a mixture containing seven synthetic standard peptides (concentration of each peptide, 111.1 μg/mL). This mixture was diluted with 50% acetonitrile (*v/v*) and 5 mM ammonium acetate in nine steps (concentration range from 1.1 to 3,704 ng/mL per peptide). The calculated concentrations agreed well with expected peptide concentrations (the arithmetic mean of accuracy was 102±26%). These results were obtained from seven replications. Coefficients of determination of calibration curves were between 0.9962 and 1.

Results and discussion

Identification of marker for milk allergens

Searching for possible markers by in silico digestion

As the sequences of the four milk proteins are known, in silico tryptic digestions of them were performed. This

facilitates the searching for peptides, which could subsequently be used as markers in MS measurements. For this purpose, 'PeptideMass' [21] was used. The sequences of milk proteins available in the database (with accession numbers P02662, α-S1-casein precursor; P02666, β-casein precursor; P00711, α-lactalbumin precursor; and P02754, β-lactoglobulin precursor) were theoretically digested with trypsin, and the masses of peptides were calculated. The obtained peptides served as candidate list for further MS experiments.

Peptide identification and characterization by MS and MS/MS

After digestion of four selected milk proteins with trypsin, the tryptic peptides in the samples were detected selectively by MS/MS. In short, the peptides, which were found by in silico digestions, were searched in MS full scan spectra of respective digested milk protein. The accurate masses of those, which were found in full scan, were determined with scan mode enhanced resolution (ER). Then, these compounds were fragmented in enhanced product ion mode (EPI) scan mode and their structures confirmed.

The analytical strategy for the peptide LIVTQTMK from BLG is described hereafter and was applied in the same way to the other tryptic peptides (data not shown). Screening and structural characterization was carried out by directly infusing diluted tryptic digests of analytical standards of milk allergen proteins into the mass spectrometer. First, MS full scan experiment of BLG was performed in the mass range of *m*/*z* 350–1,500 and 1,500–2,800 Da. MS signals of putative singly, doubly and triply charged ions of tryptic peptides, calculated from the data of the in silico digestion of the corresponding allergen proteins (in this case BLG), were searched in the MS spectra. For BLG, ten peptides were found, some of them appearing in two different charge states (Fig. 1). Also, the MS analysis of the digests of the other milk proteins yielded several ions of peptides. Subsequently, the charge states of the peptides were confirmed by individual measurements of the respective ions in the enhanced resolution (ER) scan mode (data not shown). The instrument accumulates the intact ions in the LIT and scans them to the detector at a low speed, thus providing more accurate mass values and better mass resolution than the Q1-MS full scan mode (data not shown). Then, the candidate ions were analyzed by MS/MS in enhanced product ion (EPI) scan mode, to determine their amino acid sequences. In this measurement mode of the QTrap 4000 instrument, precursor ions at a defined *m*/*z* ratio are selected in the quadrupole Q1, fragmented by collision-induced dissociation (CID) in the collision cell, the generated product ions trapped in the quadrupole Q3 operated as LIT and scanned at high sensitivity to the detector. Figure 2 shows the MS/MS product ion spectrum of the $[M+H]^+$ precursor ion at *m*/*z* 933.6, of which the entire amino acid sequence of the tryptic peptide LIVTQTMK can be deduced. As in this case, the fragmentation predominantly yielded product ions of the y-ion series; the amino acid sequence of the peptide has to be read from right to left. The tandem MS/MS data were confirmed by analysis of a peptide standard and by a theoretical MS/MS fragmentation of the putative peptide sequence carried out with the software 'Peptide Fragment Ion Analyser' (PFIA-II) [23].

In total, the structures of ten tryptic peptides of BLG were determined by MS/MS, as well as five peptides of ALA, six peptides of α-casein and five peptides of β-casein. From these peptides, a total of seven peptides (one from ALA and two for each other chosen milk protein), which exhibited the highest signal intensities in the above-mentioned LC-MS/MS experiments (in EPI mode), were selected as standard peptides for the development of an LC-MS/MS profiling method and synthesized. The synthetic peptides were purchased as lyophilized material with high purity (from 97% for LIVTQTMK to 100% for IPAVFK). They were dissolved in methanol with the concentration of 1 mg/mL as stock solutions.

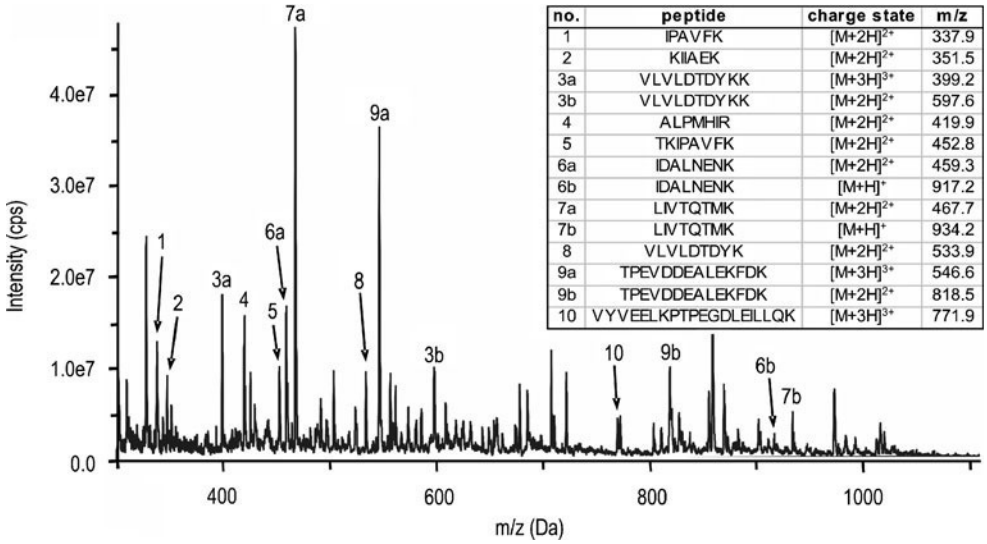
Development of an LC-MS/MS profiling method

The establishment of a profiling method is described here only for the tryptic peptide LIVTQTMK of BLG. It was carried out in the same way for the other six peptides. For this purpose, the MS/MS analysis mode 'Selected Reaction Monitoring' (SRM) of the QTrap 4000 was employed. The SRM chromatogram represents only product ions of a particular *m*/*z* ratio, which have been generated by fragmentation of a selected precursor ion. Therefore, this method has a very high specificity and sensitivity for the respective compound.

Analyte-dependent SRM parameters were optimized by direct infusion of synthetic peptides into the mass spectrometer. After combining SRM parameters with an LC gradient program, a selective LC-MS/MS method was obtained that allowed the specific determination of one or more analytes. This method was extended to a multianalyte method by including the SRM parameters for the individual tryptic peptides of the milk allergens listed in Table 1. Each analyte can be identified by using three specific SRM transitions, attaining their signal maximum at the same retention time. For each of the peptides, the most intense SRM transition served as quantifier (Q), whereas the second and third SRM transitions were chosen as qualifiers for the identification of the target compound.

Determination of species specificity by BLAST search

Since the same or closely homologous proteins and their variants are present in milk of different ruminant species—



no.	peptide	charge state	m/z
1	IPAVFK	$[M+2H]^{2+}$	337.9
2	KIIAEK	$[M+2H]^{2+}$	351.5
3a	VLVLDTDYKK	$[M+3H]^{3+}$	399.2
3b	VLVLDTDYKK	$[M+2H]^{2+}$	597.6
4	ALPMHIR	$[M+2H]^{2+}$	419.9
5	TKIPAVFK	$[M+2H]^{2+}$	452.8
6a	IDALNENK	$[M+2H]^{2+}$	459.3
6b	IDALNENK	$[M+H]^{+}$	917.2
7a	LIVTQTMK	$[M+2H]^{2+}$	467.7
7b	LIVTQTMK	$[M+H]^{+}$	934.2
8	VLVLDTDYK	$[M+2H]^{2+}$	533.9
9a	TPEVDDEALEKFDK	$[M+3H]^{3+}$	546.6
9b	TPEVDDEALEKFDK	$[M+2H]^{2+}$	818.5
10	VYVEELKPTPEGDLEILLQK	$[M+3H]^{3+}$	771.9

Fig. 1 MS full scan spectrum of a tryptic digest of BLG, directly infused into the mass spectrometer, containing diagnostic peptide ions, which were listed in the table (*insert*). The charge states of the peptides have been confirmed by MS measurements in the enhanced resolution (ER) scan mode

for example, cow milk caseins occur with 80% to 90% sequence homologies in the other species as well [1]—a BLAST search was applied (BLAST 2.2.18, first access 28.10.2008) [22] in order to determine the species specificity of these selected tryptic peptides.

The results of BLAST search for all of the seven peptides are summarized in Table 2. The BLAST search showed that all the peptide sequences were found in *Bos taurus* (cattle). The peptide sequence FFVAPFPEVFGK of α-casein appeared additionally in α-casein of *Bubalus bubalis* (water buffalo). YLGYLEQLLR, also from α-casein, was found in α-casein of cattle, water buffalo, *Capra hircus* (goat) and *Ovis aries* (sheep). Similarly, GPFPIIV from β-casein was found in β-casein of water buffalo as well as in cattle. The second selected peptide of β-casein, VLPVPQK, seems to be a highly conserved region in mammalians protein, as it was found in β-casein of cattle, water buffalo, goat, sheep, *Bos indicus* (zebu), *Giraffa camelopardalis* (giraffe) and even in *Tragulus napu* (greater mouse-deer), a species of even-toed ungulate which is found in Southeast Asia.

Like VLPVPQK, the peptide VGINYWLAHK from ALA could be found in ALA of various species, e.g. cattle, water buffalo, goat, sheep, zebu, *Bos grunniens* (yak) and also in *Cervus canadensis xanthopygus* (Manchurian wapiti), a subspecies of elk, native to eastern Asia. The peptide IPAVFK from BLG was found in BLG of cattle, water buffalo, goat, sheep, and *O. aries musimon* (European mouflon). However, LIVTQTMK, also from BLG, seems to be cattle specific and could not be found in the other species. These results are data bank-dependent; currently, no

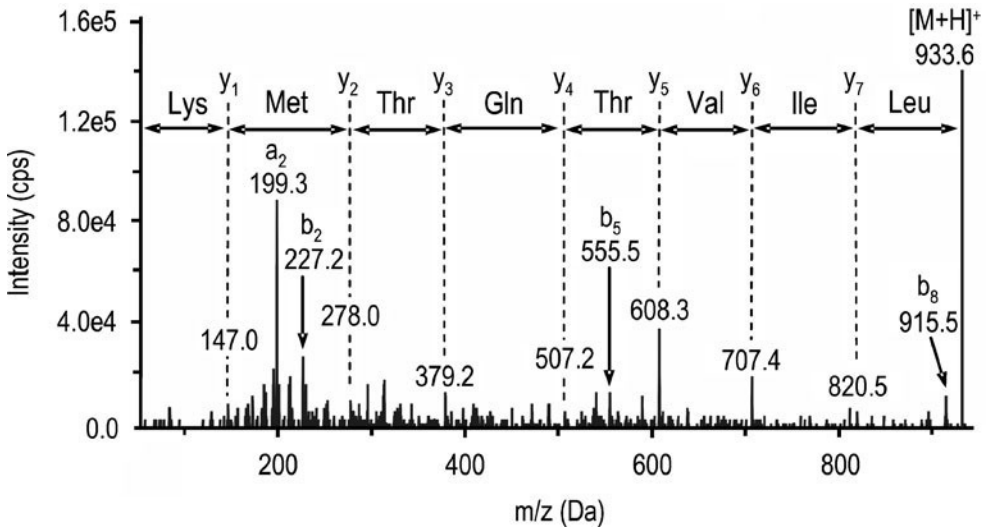


Fig. 2 MS/MS spectrum of the tryptic peptide LIVTQTMK from BLG. As fragment ions of the y-ion series occur in the spectrum, the amino acid sequence must be read from right to left

Table 2 The results of BLAST search for selected standard peptides of four milk allergens

Species	α-Lactalbumin	β-Lactoglobulin		α-Casein		β-Casein	
	VGINYWLAHK	LIVTQTMK	IPAVFK	FFVAPFPEVFGK	YLGYLEQLLR	VLPVPQK	GPFPIIV
Cattle	√	√	√	√	√	√	√
Water buffalo	√		√	√	√	√	√
Goat	√		√		√	√	
Sheep	√		√		√	√	
Zebu	√					√	
Giraffe						√	
Greater mouse-deer						√	
Yak	√						
Manchurian wapiti	√						
Mouflon			√				

Only accessions with 100% query coverage were considered

more proteins could be found, but the data bank should be searched routinely to find out if there are any new entries.

Consequently, LIVTQTMK was the only peptide which could be used as a specific marker for detection of cow milk in the samples. The two other peptides, namely, GPFPIIV from β- and FFVAPFPEVFGK from α-casein, could be used as marker for detection of milk from cow and water buffalo; however, the probability of contamination of cow milk with water buffalo milk is low. The other peptides could be used as markers for identification of ruminant milk in general, where cow milk is one of them.

Allergenicity studies have shown the existence of numerous allergenic IgE and IgG binding epitopes, which are widely spread along each of the four milk allergens [1, 3–6, 16]. All of our seven selected peptides are within these epitopes, for example, a study carried out on sera of 46 patients allergic to milk showed that LIVTQTMK and IPAVFK, both from BLG, could be recognized by 65.4% and 28.3% of the sera, respectively [5].

Additionally, it has to be taken into consideration that the LC-MS/MS method is not able to distinguish between isobaric peptides such as LIVTQTMK from cattle and IIVTQTMK from European mouflon. Both of them have a molecular weight of 933.54 Da, but differ in their sequences due to the isobaric amino acids Leu and Ile in the first position.

Identification of milk allergens in food samples by the LC-MS/MS profiling method

In calibration analysis with synthetic peptide standards, the lowest determined concentrations were between 1 and 11 ng/mL (signal-to-noise ratio=3; Table 1), dependent on the peptide. For most of the peptides, e.g. for the BLG-derived peptide LIVTQTMK, the lowest concentration was found to be 1.11 ng/mL; the linear range extends up to 1,111 or 3,704 ng/mL, respectively. Only for VGINYW-LAHK (from ALA), the concentration was as high as 11.1 ng/mL; it exhibited poor SRM signal response, although it showed through five candidate peptides from α-lactalbumin the highest signal intensities in the MS full scan to MS/MS product ion scans prior to purchase of standards.

Using these synthetic peptides and their calibration curves, the presence and also the concentration of natural milk proteins in different food samples could be determined (Table 3). First, samples like milk and dairy products (yoghurt, various different cheese sorts, e.g. parmesan and mozzarella and whey drink) and also milk powder were used to develop and establish the method. At the next step, the method was applied to different samples as yoghurt from sheep's milk and chocolate samples, where milk belonged to ingredients such as country crisp, milky bites, nutella and Neapolitan wafer, and also blank samples, for example, sponge fingers, peanut puffs and ice cream cones. The samples were diluted to different concentrations before being analyzed with the LC-MS/MS method in SRM mode, which consisted of parameters for the seven tryptic peptides specifying four milk proteins. Characteristic peak patterns (profiles) were obtained, indicating significant differences between the concentrations of the individual peptides in the samples (Fig. 3).

The method showed good sensitivity, and the results could be repeated. All of the selected peptides could be detected and measured with a concentration down to 1 ng/mL in milk powder, milk and dairy products, as expected; in cheese varieties and also in yoghurt, the lowest detectable concentration of whey proteins is lower than casein proteins as estimated. It seems that fermentation did not disturb the detection. In whey drink, all tryptic peptides

Table 3 Lowest detectable concentration for different food samples and blanks

Food samples (replications)	Lowest detectable concentration (ng/mL)						
	ALA	BLG		α-Casein		β-Casein	
	VGINYWLAHK	IPAVFK	LIVTQTMK	YLGYLEQLLR	FFVAPFPEVFGK	GPFPIIV	VLPVPQK
Milk (6)	3.8	3.4	1.0	3.0	1.1	2.1	1.2
Yoghurt (5)	9.6	5.3	7.6	23.9	21.2	16.3	10.1
Mozzarella (4)	1.3	3.2	4.4	198.0	182.0	114.0	68.8
Parmesan (4)	15.5	5.0	1.1	12.4	7.6	5.3	3.3
Whey drink (4)	17.3	2.4	2.8	1.1	1.8	1.8	1.5
Milk powder (4)	21.5	11.2	10.2	41.8	82.4	57.3	31.6
Sheep's yoghurt (2)	14.0	4.4	15.7	25.2	2.8	25.0	26.5
Country crisp (4)	17.7	9.4	10.4	42.8	47.6	40.0	22.8
Milky bites (3)	5.8	3.3	2.5	14.7	16.2	12.1	6.0
Hazelnut spread (2)	No peak	3.9	1.1	4.4	7.5	4.3	2.2
Neapolitan wafer (2)	11.1	7.7	4.9	20.8	22.3	16.2	5.5
Sponge fingers (2)	No peak	No peak	No peak	No peak	1.5	2.0	1.2
Peanut puffs (2)	No peak	No peak	No peak	No peak	No peak	No peak	No peak
Ice cream cones (2)	No peak	No peak	No peak	1.6	1.4	No peak	1.2

The number of replications was given in parentheses. The measurements were performed in different dilution

of whey and casein proteins could be detected with the concentration down to 1.1 ng/mL. The lowest detectable concentration for milk powder is much higher than for milk. It could be based on the fact that milk powder could not be resuspended well in aqueous buffer. All of the peptides could be detected in sheep's yoghurt, too. However, regarding BLAST search (Table 2), three of seven selected milk peptides should not appear in sheep

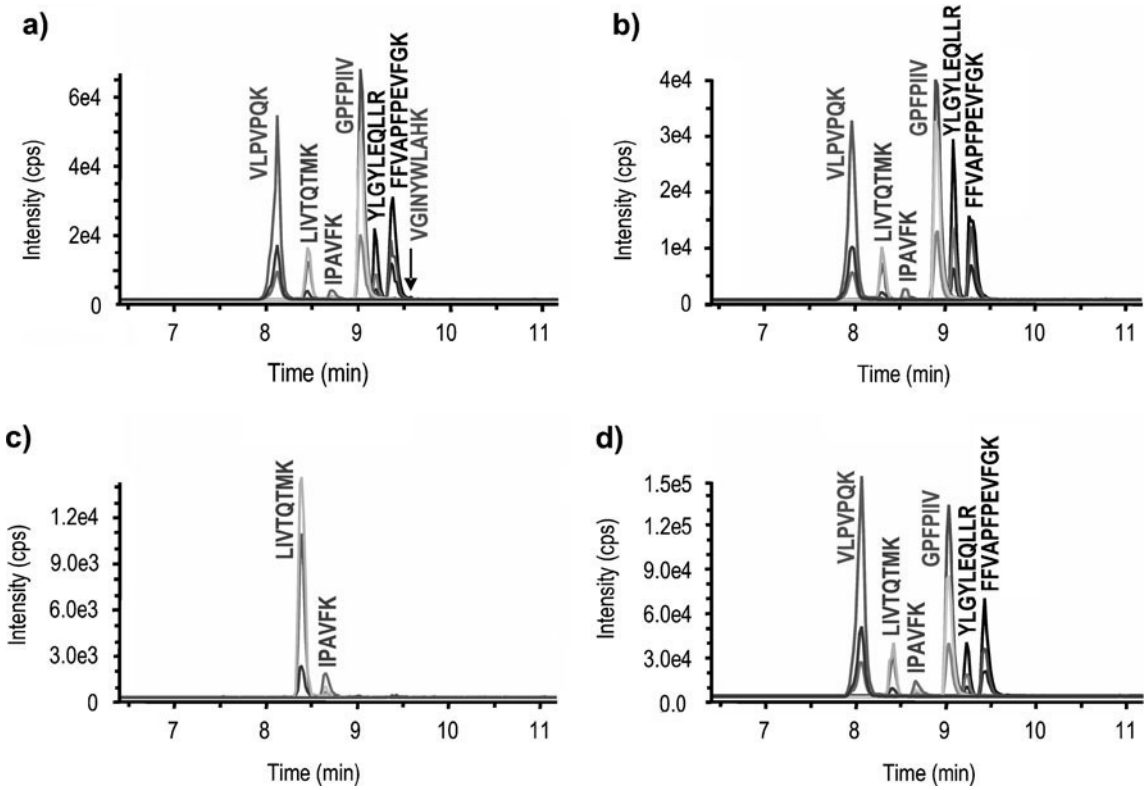


Fig. 3 Profiling of milk (**a**) and three milk-containing food samples: (**b**) parmesan, (**c**) whey drink and (**d**) yogurt by LC-MS/MS in SRM mode

milk. It could be evidence either for food adulteration (mixing cow milk with sheep) or cross-contamination during production.

In milk chocolate samples, again all peptides could be determined with the lowest detectable concentration down to 1.1 ng/mL. Only VGINYWLAHK from ALA could not be detected in hazelnut spread. It seems that with this method also, the detection of probably masked proteins by polyphenols is possible compared to immuno-based methods, where special extraction efforts have to be taken into consideration for extraction of proteins from samples including chocolate to avoid protein loss by polyphenols appearing in chocolate.

Although in the ingredients of sponge fingers and ice cream cones no milk was listed, in both of them, three peptides could be detected, however, in low concentrations; it could be an evidence of cross-contamination during processing. Only peanut puffs proved to be a real blank sample, which was further used for spiking experiments.

Recovery calculation for spiked samples

The spiking experiments could be divided into two parts: standard addition and recovery calculations. The standard addition of peptides mixtures to food samples was used to test the matrix effect. For this purpose, at first, all 14 digested food samples as well as four used concentrations of peptide mixtures were separately measured by MS, and then the spiked samples after standard addition. The standard additions were calculated by dividing the calculated concentrations of spiked samples (from peak area) by the sum of the concentrations of peptide mixture and food samples (Table 4a). For calculating the results in Table 4,

Table 4 Spiked samples

	ALA	BLG		α-Casein		β-Casein	
Food and blank samples	VGINYWLAHK	IPAVFK	LIVTQTMK	YLGYLEQLLR	FFVAPFPEVFGK	GPFPIIV	VLPVPQK
				Standard addition (%)			
Milk[a]	77±2	85±5	97±0	106±4	92±7	95±0	98±0
Yoghurt[a]	82±2	87±0	85±20	71±1	99±1	88±4	95±4
Mozzarella[a]	80±20	82±12	85±23	97±5	98±2	97±1	92±9
Parmesan[a]	87±6	79±10	84±15	91±13	92±5	93±4	86±4
Whey drink[a]	68±7	69±13	81±9	96±10	96±4	93±8	84±10
Milk powder[a]	78±5	94±0	103±4	112±13	96±3	97±3	92±7
Sheep's yoghurt[a]	83±11	77±13	105±8	91±9	92±6	97±1	92±3
Country crisp[a]	70±3	92±4	85±7	101±5	92±3	87±1	84±3
Milky bites[a]	82±5	79±21	69±7	92±7	95±4	96±1	83±4
Hazelnut spread[a]	86±7	80±23	70±16	85±11	88±9	87±6	87±7
Neapolitan wafer[a]	89±13	74±10	78±10	80±9	96±3	96±5	85±9
Sponge fingers[a]	95±6	76±4	72±21	85±2	94±4	84±3	89±6
Peanut puffs[a]	91±9	50±1	54±3	84±12	93±8	95±2	95±1
Ice cream cones[a]	89±5	80±7	57±25	70±14	89±0	90±5	84±4
				Recovery (%)			
Blank extract spiked with milk[b]	Not possible	98±10	52±1	102±1	97±6	89±4	72±5
Blank extract spiked with peptides mixture[b]	59±7	93±6	80±3	102±7	112±5	128±12	79±5
Blank spiked with 1 ml of milk[c]	89±4	80±22	72 ±11	103 ±15	109±4	94±4	98±5
Blank spiked with 500 μl of milk[c]	92±11	129±4	102±1	110±7	128±10	98±8	113 ±1
Blank spiked with peptides mixture[c]	89±7	100±11	90±12	99±15	98±9	129±14	101±2

[a]Standard addition; four concentration of peptide mixtures were added to the food samples after digestion (the mean values of four concentrations are given in the table)

[b]Blank sample (peanut puffs) spiked with milk or peptides mixture after extraction, before digestion

[c]Blank sample (peanut puffs) spiked with milk or peptides mixture before extraction

The recoveries were calculated from two replications (for b and c)

all spike levels were considered, and arithmetic means were calculated. The results were satisfying because all the peptides were found again with high percentage (over 50%).

Alternatively, to determine the influence of extraction and digestion procedure, the selected blank sample (peanut puffs) was spiked with either milk or peptide mixture, directly before and after extraction, then digested with trypsin, measured with MS and again recoveries were calculated (Table 4b). The calculations were performed by dividing the concentrations of spiked samples (measured by peak area) by the sum of the concentrations of blank (which should be zero) and milk, or peptide mixture, respectively.

The calculated recoveries for spiked blank samples showed that the digestion procedure only has an influence on the already identified critical peptide VGINYWLAHK from ALA, as this was the only peptide which was not detectable. The recoveries for the other peptides with over 52% were satisfying. The measurements including the extraction procedure also showed very good reproducibility from 72 to 129% for all peptides which indicates that in this case, the extraction procedure and the tryptic digest do not have a negative influence on the following MS measurements, and there are no losses of peptides detectable; there is also no significant difference between spiking with milk or peptides mixture here.

Conclusion

Seven peptides (chain length, 5–12 amino acids) derived from tryptic digestion of four allergenic milk proteins were selected, according to data obtained from evaluation of MS/MS spectra, as potential markers for the detection of these proteins (α-casein, β-casein, α-lactalbumin and β-lactoglobulin) in food samples. The BLAST search has shown that only one peptide (LIVTQTMK from BLG) could be used as a specific marker for detection of cow milk in the samples; two other peptides, FFVAPFPEVFGK (α-casein) and GPFPIIV (β-casein), could be used as marker for detection of milk from cow and water buffalo; and four other peptides could be used as markers for identification of ruminant milk in general. Using these peptides as the standards, an LC-MS/MS multianalyte method based on SRM for the simultaneous determination of the peptides was developed, optimized and applied to tryptic digestion of different dairy and processed food samples, and also to blanks and spiked samples. The results showed that the method was able to detect the selected peptides in samples with concentrations down to 1 ng/mL and yielded peak patterns characteristic for the individual samples; however, for further trace amount determination, the method has to undergo a complex validation procedure.

The HPLC method itself is very rapid as the run lasts only 20 min. The LC-MS/MS in combination with the selected reaction monitoring (SRM) is a very selective method with good quantification properties. Although sample preparation is more complex compared to immuno-based assays, it could be shown by spiking experiments that the extraction and digestion procedures do not have a negative influence on the results. Therefore, the used LC-MS/MS method could be a possible candidate in the process of reference method search for food allergen detection.

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3.5 Marker peptide selection for the determination of hazelnut by LC–MS/MS and occurrence in other nuts

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Marker peptide selection for the determination of hazelnut by LC–MS/MS and occurrence in other nuts

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Abstract The aim of this work was identifying and selecting hazelnut marker peptides and subsequently developing a complementary method of common immunoassay for the detection of hazelnut. For this purpose, at first, an in silico digestion of three major hazelnut allergens (Cor a 8, Cor a 9 and Cor a 11) was performed to get information about expected peptides. After extraction and trypsin digestion of hazelnut proteins, the samples were measured with tandem mass spectrometry (MS/MS) by direct infusion, which led to identification of 14 peptides. Eight of them with the highest MS signal were synthesized and used as standards for developing a liquid chromatography (LC)–MS/MS method in selected reaction monitoring (SRM) mode. Since almost all food allergens derived from nuts belong to the seed storage protein family and have homologue structure, a Basic Local Alignment Search Tool (BLAST) search was performed to identify the hazelnut specificity of the developed method. According to BLAST, only one peptide occurs in three other nuts, and the remaining seven selected peptides are hazelnut specific. Additionally to hazelnut, the eight other listed nuts in Directive 2003/89/EC as allergen were extracted, digested and measured with the developed method to prove the BLAST results. The analytical data confirmed that six peptides are hazelnut specific, on the contrary to anti-hazelnut antibodies, which showed cross-reactivities to all other nut extracts. Comparing these results, it could be shown that with this LC–MS/MS method in SRM mode, the specific detection of hazelnut is possible.

Keywords Nut allergens · Cor a 8 · Cor a 9 · Cor a 11 · Hazelnut · LC–MS/MS

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Introduction

Because of its special nutritional values, hazelnut (HN) is often used in human nutrition with a special emphasis in ice cream, chocolates and confectionary products such as nougat [1]. However, its proteins are in discussion as they can cause pollen- and food-related allergy. At least five protein types of hazelnut appear to be involved in these allergic reactions. The first major allergen, Cor a 1 (18 kDa, Bet v 1 family), is a typical pollen allergen. The second one, Cor a 2 (14 kDa), belongs to the profilin family and could be found in pollen as well as in seed. The other three belong to the seed storage protein family. Cor a 8 (9 kDa) is a nonspecific lipid transfer protein from the prolamin superfamily [2], which is associated with severe allergic reactions to hazelnuts. The other major food allergen present in hazelnut is Cor a 9 (Corylin), a 59 kDa protein which belongs to the legumin (11S globulin protein family). The last major food allergen from HN is Cor a 11 (48 kDa); as glycosylated vicilin, it belongs to the 7S seed storage globulin family [3].

It has to be stated that most of the listed major tree nut allergens are from the seed storage family such as legumines, 2S albumins and vicilins. For example, the major allergens Ara h 1 (peanut), Jug r 2 (walnut), Ana o 1 (cashew) and Cor a 11 (hazelnut) are vicilins (7S globulins) [3]. Recently, Bignardi et al. [4] investigated the detection

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of the 11S globulin family from five different nuts—Ana o 2 (cashew), Cora a 9 (hazelnut), Pru 1 (almond), Jug r 4 (walnut) and Ara h3/4 (peanut)—in breakfast cereals and biscuits by liquid chromatography (LC)–linear ion trap–tandem mass spectrometry (MS/MS). Although there is homology between 11S globulin proteins from different legumes, this sequence homology is more definitive for 7S globulin proteins, where the variable domains are mainly found within the N- and C-terminal regions and not in the inside parts of the sequences, whereas in 11S proteins, only β-polypeptide is conservative and the α-polypeptide is variable [5]. This homologous structure can cause allergic cross-reactivity among these nuts; however, it is not a guaranty for cross-reactivity [6]. Goetz et al. [6] could show strong cross-reactivity of hazelnut to walnut and pecan, and moderate cross-reactivity to cashew, Brazil nut, pistachio and almond.

Since one third of all anaphylactic reactions were caused by tree nut ingestion [6], a major focus of allergenic food protein analysis is the detection and characterization of nuts listed in Annex IIIa of the EC-Directive 2007/86/EC, in which the indication of the ingredients present in foodstuffs was requested, especially those that cause allergies or intolerances in consumers. The list includes almonds (*Amygdalus communis* L.), hazelnuts (*Corylus avellana*), walnuts (*Juglans regia*), cashews (*Anacardium occidentale*), pecan nuts (*Carya illinoinensis*), Brazil nuts (*Bertholletia excelsa*), pistachio nuts (*Pistacia vera*) and macadamia nuts (*Macadamia ternifolia*) [7]. A lot of research was done on allergen purification, characterisation [2, 3, 8] and expression of allergenic proteins [9], but for the determination of the hazelnut content in different food, efficient and reliable quantification and detection methods are needed. The immunochemical methods, which use specific antibodies for the detection of proteins, are mostly employed because antibodies can recognise very specific proteins in trace amounts in a complex food matrix [10]; however, the food analyst must be aware of the class of antibody being used in an immunoassay [11] and the used immunogen, as cross-reactivities can occur. Additional valuable information can be found in the botanical family tree of the eudicots. Walnut and pecan belong to Juglandaceae. Cashew and pistachio belong to Anacardiaceae. Hazelnut belongs to the family Betulaceae, which explains cross-reactivities to birch. Almond belongs to the family Rosacea, and all together, they appear within the rosids, which are beside the asterids the largest clades in the eudicots. As hazelnuts belong beside walnut and pecan to the same botanical order, cross-reactivities within them can occur. Therefore, antibodies have to be very well characterized according to their properties. This highlights the need for comparative methods, which are still scarce in the field of food allergen detection [12]. Besides rapid immunoanalytical methods, advanced analytical methods based on LC–MS/MS are highly requested for structural investigation and quantification of allergenic proteins [13].

The objective of this study was in first instance extracting and digesting hazelnut proteins from processed hazelnuts in order to characterise and identify some marker peptides for further development of a complementary method to immuno-based assays for food analysis with a different technique. Since it has been proven that cross-reactivities especially can occur in tree nuts, where botanical family associations are given [6], in this work, in-house-produced anti-HN antibodies were not used for assay development but for immunoblotting experiments to characterise the cross-reactivities of the antibodies to other nut extracts, which can be found within the labelling list of the EC-Directive. The protein extracts of different nuts were detected by western blot (using two in-house-produced antibodies) as well as digested with trypsin. The sequence homology and specificity of target hazelnut peptides were searched with the Basic Local Alignment Search Tool (BLAST) [14] and compared with the protein sequences from numerous other organisms and within the rosid family. Digested hazelnut extract was measured by MS/MS, and subsequently, an LC–MS/MS method in selected reaction monitoring (SRM) mode was developed. Additionally, the other nut extracts were measured by this LC–MS/MS method. This leads to interesting differences in antibody detection and MS/MS measurements.

Materials and methods

Materials, chemicals and reagents

The nuts (hazelnut, walnut, pecan, pistachio, peanut, cashew, Brazil nut, macadamia and almond) were purchased from local markets.

NuPAGE® 12% Bis–Tris pre-cast gels (1.0 mm × 15 well), See Blue® Plus2 prestained standard, MagicMark™ XP Western Standard, NuPAGE® LDS sample buffer and Simply Blue™ SafeStain were purchased from Invitrogen Corporation (Carlsbad, CA, USA).

The anti-HN antibodies (polyclonal from rabbit and monoclonal from mouse) were produced in-house. The labelled species-specific secondary antibody for western blot (anti-mouse-IgG-HRP and anti-rabbit-IgG-HRP) were obtained from Sigma-Aldrich (Steinheim, Germany) as well the chemicals ammonium acetate (MS grade), tris[hydroxymethyl]aminomethane (Trizma® base, 99.9%), albumin bovine serum (fraction V, ≥96%), ethylenediamine-tetraacetic acid (EDTA, 99%), Tween20, hydrogen peroxide (30 wt.%, semiconductor grade), 3,3′,5,5′-tetramethylbenzidine (TMB, 89%), Bis(2-ethylhexyl) sulphosuccinate sodium salt (DONS)

and iodoacetamide. Ammonium hydrogen carbonate (≥99%), acetonitrile (HPLC gradient grade), sodium dodecyl sulphate (SDS, ≥99%), 2-(*N*-Morpholino)ethanesulphonic acid (MES, PUFFERAN® 99%) and 1,4-dithiothreitol (≥99%) were purchased from Carl Roth GmbH (Karlsruhe, Germany). Trypsin (Sequencing Grade, modified) was from Roche Applied Science (Mannheim, Germany). Urea, acetic acid (glacial) 100%, citric acid, sodium chloride (GR for analysis), sodium dihydrogen phosphate monohydrate (GR for analysis) and disodium hydrogen phosphate dihydrate were from Merck (Darmstadt, Germany). Ethanol 99%, denaturated, was from J.T. Baker chemicals B.V. (Deventer, Holland).

The eight selected peptides (one from Cor a 8, four from Cor a 9 and three from Cor a 11), which showed the highest MS signal, were ordered in lyophilized form from "Peptide 2.0" (Chantilly, VA, USA).

NuPAGE® MES SDS Running Buffer, 20×, was prepared by dissolving 60.6 g Trizma® base, 97.6 g MES, 10 g SDS and 3.0 g EDTA in 500 mL water. For electrophoresis, stock solution was diluted to 1× with water just before use.

Phosphate-buffered saline (PBS), 0.2 M, pH 7.5, was prepared by dissolving 32.22 g Na_2HPO_4, 2.62 g NaH_2PO_4 and 21.18 g NaCl in 1,000 mL distilled water. For wash solution PBST, 0.1% Tween 20 was added to PBS buffer.

Substrate solution for western blot included 24 mg TMB, 80 mg DONS, 10 mL ethanol and 30 mL citrate buffer (0.15 M, pH 5.0). Per 10 mL of substrate solution, 5 μL H_2O_2 was added short before beginning of the reaction.

The used water was either purified by reverse osmosis or drawn from a Milli-Q plus System from Millipore (Molsheim, France). If not stated otherwise, procedures were performed at room temperature.

Sample preparation (extraction and digestion)

The nuts were ground and mixed with 0.1 M NH_4HCO_3, pH 7.6 (1:10). The extraction was performed with a rotary shaker at 50 rpm and at 4 °C for 2 h. The mixture was centrifuged for 30 min at 6,500×*g* and 4 °C using a Conical Tube Fixed-angle Rotor (C1015) and an ALLEGRA® X-22 centrifuge (Beckman Coulter, Inc., Fullerton, CA, USA). The supernatant was filtered through glass fibre MN85/70 BF from Macherey-Nagel (Macherey-Nagel GmbH & Co., Düren, Germany) and cellulose acetate membrane filter, 0.2 μm (Sartorius Stedim Biotech GmbH, Goettingen, Germany). The filtrate was dried completely overnight using a vacuum centrifuge, Model VR-1/120/240 (Heraeus Instruments, Denmark).

Proteolytic digestions with trypsin were carried out using a protocol for in-solution digestion [15] with some modifications; in short, the extracted proteins were resuspended in 6 M urea, 0.1 M NH_4HCO_3 buffer. One hundred-microlitre aliquots were reduced and alkylated using 0.2 M DTT and 0.2 M iodoacetamide in 0.1 M NH_4HCO_3. Twenty micrograms of trypsin was added to the samples and incubated at 37 °C overnight. The reaction was stopped by addition of acetic acid.

SDS-PAGE and western blot

Extracted proteins were separated via gel electrophoresis. Discontinuous SDS-polyacrylamide gel electrophoresis (PAGE) was performed using Invitrogen pre-cast NuPAGE® 12% Bis–Tris Gels and MES SDS running buffer, following the manufacturer's instructions. The proteins were either visualized with Coomassie staining (with SimplyBlue™ SafeStain according to manufacturer's recommendations) or blotted (western blot).

For blotting, the XCell II™ Blot Module (Invitrogen Corporation, USA) was used. The proteins were transferred to a Protran® 0.45 μm nitrocellulose membrane (Schleicher & Schuell BioScience, Dassel, Germany) and blocked with 2% bovine serum albumin. After washing with PBST (2× 10 min), the membrane was incubated with primary antibodies, which were in-house-produced anti-hazelnut antibodies. Another wash step (3×10 min with PBST) was followed before incubation with the secondary anti-species antibodies, which were linked to horseradish peroxidase (HRP) and used for the colorimetric detection of the proteins. The reaction of hydrogen peroxide (included in substrate solution) with peroxidase converts the soluble dye into an insoluble form. It precipitates next to the enzyme and thereby stains the proteins on the membrane.

Liquid chromatography–tandem mass spectrometry (LC–MS/MS)

The chromatographic separation of tryptic-digested samples was carried out on an Aquasil C18 reversed phase column (50×2.1 mm, 3 μm particle size from Thermo Electron Corporation, Marietta, GA, USA), equipped with a C18 4× 3 mm i.d. security guard cartridge (Phenomenex, Torrance, CA, USA), using an 1100 series HPLC (Agilent, Waldbronn, Germany).

Injection volume was 5 μL, and the flow rate was 0.5 mL/min. Eluent A consisted of 10% acetonitrile, 5 mM ammonium acetate, and eluent B was 95% acetonitrile, 5 mM ammonium acetate. The HPLC conditions were initial hold of 0.5 min, linear gradient from 3% to 40% B within 11.5 min, rapidly up to 100% B and 4 min hold, switch back to 3% B within 2 min and equilibration for 5 min. MS measurement was done between 4 and 14 min.

A QTrap 4000 LC–MS/MS system (Applied Biosystems, Foster City, CA, USA), equipped with a Turbo V Ion Spray (ESI) source was used. Screening and identification

of tryptic peptides, as well as optimization of SRM parameters for profiling of selected tryptic peptides, was carried out by directly infusing tryptic-digested hazelnut proteins or peptide standard solutions, respectively, into the mass spectrometer at a flow rate of 10 μL/min. Chromatographic and (tandem) mass spectrometric data were evaluated using the Analyst™ software version 1.5.

The used ESI source parameters were positive ionization mode; curtain gas, 20 psi; both ion source gas 1 and 2, 50 psi; source temperature, 150 °C for directly infusion and 535 °C for LC–MS/MS; and ion spray voltage, +4,000 V. Collision gas (nitrogen) was set to high. For each SRM transition, a dwell time of 50 ms was chosen, and the pause between mass ranges was 5 ms. Optimized SRM parameters (Table 1) were used for profiling the tryptic peptides of hazelnut allergens by LC–MS/MS.

Quantification of peptides in SRM mode

For the determination of the peptide concentrations in the tryptic-digested samples by the LC–MS/MS method in SRM mode, linear working ranges were evaluated. Calibration curves were created by analyzing eight dilution levels of a mixture containing eight synthetic standard peptides (concentration of each peptide, 125 μg/mL). This mixture was diluted with 10% acetonitrile and 5 mM ammonium acetate in nine steps (concentration range from 3.1 to 4,166.7 ng/mL per peptide).

Results and discussion

Gel electrophoresis and western blot

The gel electrophoresis is an important part of proteomics analysis because in one single step, a complex mixture of proteins can be separated. Staining reveals information about characteristic band distribution of the applied extracts and protein mixtures throughout the used separation gel. An additional blotting step offers the advantage using an antibody–antigen reaction for further characterisation of the blotted proteins with the developed antibodies.

Table 1 The parameters of the LC–MS/MS method in SRM mode for profiling of tryptic peptides of hazelnut allergens

Protein	Tryptic peptide	MW (Da)	Q1 (*m/z*)	DP (V)	Q3 (*m/z*)	Q3 ion	CE (V)	
Cor a 8	GIAGLNPNLAAGLPGK	1,461.8	732.2	61	171.3	b_2	47	q_2
					301.3	y_3	33	Q
					937.7	y_{10}	50	q_1
Cor a 9	ALPDDVLANAFQISR	1,628.9	815.6	76	175.2	y_1	63	q_2
					185.2	b_2	87	q_1
					906.6	y_8	47	Q
	QGQVLTIPQNFAVAK	1,612.9	807.8	71	186.2	b_2	69	q_2
					314.2	b_3	51	q_1
					874.6	y_8	37	Q
	INTVNSNTLPVLR	1,439.8	721.1	66	228.2	b_2	51	q_1
					484.4	y_4	35	Q
					1,013.7	y_9	35	q_2
	WLQLSAER	1,001.5	501.9	56	159.2	a_1	45	q_1
					272.3	a_2	29	Q
					575.5	y_5	27	q_2
Cor a 11	AFSWEVLEAALK	1,362.7	682.7	61	191.3	a_2	43	Q
					402.3	y_4	41	q_1
					644.4	y_6	31	q_2
	LLSGIENFR	1,047.6	524.9	56	199.3	a_2	29	Q
					565.4	y_4	25	q_2
					822.5	y_7	27	q_1
	ELAFNLPSR	1,045.6	524.0	56	215.2	a_2	35	q_1
					359.3	y_3	25	Q
					586.4	y_5	27	q_2

These peptides were used as standards for calibration curve. All of the precursor ions were doubly charged ($[M+2H]^{2+}$)

MW neutral experimental mass of peptide, *Q1* precursor ion, *DP* declustering potential, *Q3* product ion, *CE* collision energy, *Q* quantifier, q_1 first qualifier, q_2 second qualifier

In this work, at first, the profiles of proteins and differences between nut extracts were visualised by Coomassie staining (Fig. 1a), and then western blot provides analytical approaches for protein characterisation of prepared extracts. Nut extraction was done with NH_4HCO_3 at a neutral pH (7.6). Figure 1a shows the obtained profiles of the different nuts. In the molecular weight range from 70 to 30 kDa, where 11S and 7S seed storage proteins can be found, different bands were visualized, but also in the low molecular weight range below 17 kDa, proteins can be detected. As expected, anti-hazelnut antibodies showed cross-reactivity to almost all of different nut extracts. In case of the polyclonal antibody (Fig. 1b), which was raised against a whole hazelnut protein extract, several proteins within all nut extracts could be detected across the whole molecular weight range. Even the monoclonal anti-hazelnut antibody (Fig. 1c) showed cross-reactivity to different proteins of nut extracts. However, it seems as the reaction of the monoclonal antibody is more specific to the proteins in the molecular weight range between 50 and 40 kDa. This would be the region of the 7S storage proteins, which have their variable domains mainly located in the outer part of the proteins. However in contrast to Goetz et al., where a strong cross-reactivity between walnut, pecan and hazelnut was observed, our tested monoclonal anti-hazelnut antibody showed strong cross-reactivity to almond, Brazil nut and pistachio and moderate cross-reactivity to the other nuts. The reason of the high recognition pattern of the anti-hazelnut antibodies could be the fact that most of these nut allergens are seed storage proteins and some of these nuts are from the same botanical family and order, and additionally, the antibodies were raised against a complex protein mixture.

Identification of hazelnut allergen marker peptides by MS

In silico digestion

For facilitating the search of possible hazelnut marker peptides, in silico tryptic digestions of hazelnut protein sequences were performed as described earlier for milk [16]. The software tool "PeptideMass" [17] was used for in silico trypsin digestion of three hazelnut allergen sequences available in the UniProt database (Universal Protein Resource) [18] with accession numbers Q9ATH2 (Cor a 8), Q8W1C2 (Cor a 9) and Q8S4P9 (Cor a 11). The obtained peptides (data not shown) were further used for marker search by MS.

Identification of hazelnut marker peptides by MS/MS

After tryptic digestion of hazelnut extracts, a MS full scan was performed, and the obtained peptides from in silico digestion were sought. The next steps were enhanced resolution mode (ER), where the accurate masses of peptides found in full scan were determined, followed by enhanced product ion mode (EPI), where their compounds were fragmented and the amino acid sequences of the peptides were deduced. The MS/MS data were confirmed by in silico fragmentation of peptides using the software "Peptide Fragment Ion Analyser" (PFIA-II) [19].

With this strategy, the structures of one tryptic peptide from Cor a 8, eight peptides of Cor a 9 and five peptides from Cor a 11 (each consisting of 8–17 amino acids) were determined by MS/MS (data not shown). From these peptides, a total of eight peptides with the highest signal intensities in EPI mode were synthesized (one peptide from Cor a 8, four from Cor a 9 and three from Cor a 11, listed in

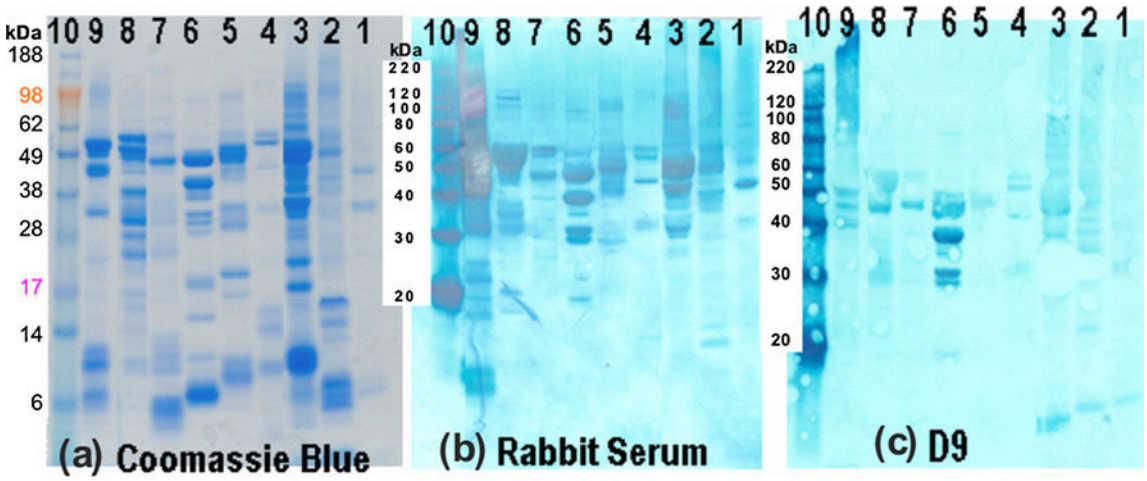

Fig. 1 Coomassie staining (**a**) and western blot of nut extracts with anti-hazelnut polyclonal rabbit serum (**b**) and monoclonal antibody D9 (**c**). *Lane 1*: walnut extract, *lane 2*: pecan extract, *lane 3*: pistachio extract, *lane 4*: peanut extract, *lane 5*: cashew extract, *lane 6*: Brazil nut extract, *lane 7*: macadamia extract, *lane 8*: almond extract, *lane 9*: HN extract and *lane 10*: MW marker

Table 1) and used as standard peptides for the development of an LC–MS/MS profiling method. They were delivered in lyophilized form with high purity (from 95.0% for QGQVLTIPQNFAVAK to 99.4% for ELAFNLPSR) and then dissolved in methanol with the concentration of 1 mg/mL as stock solutions. The purity of these stock solutions was also checked via capillary electrophoresis (Hewlett Packard G1600AX 3D CE, Hewlett-Packard Company, USA); in each stock solution, only one peak was identified. The analyses were performed at optimized CE separation conditions.

BLAST search

For the determination of hazelnut specificity of these selected tryptic peptides, a BLAST search was applied [14]. According to BLAST (BLAST 2.2.22, first access 1 March 2010), all of them are hazelnut specific except WLQLSAER from Cor a 9, which could occur, besides in hazelnut, also in *P. vera* (pistachio), *C. illinoinensis* (pecan), *Ficus pumila* var. *awkeotsang* and *J. regia* (walnut). It has to be mentioned that these results are data bank dependent; it could be possible that they occur in more plants, but probably the proteins of these plants are not investigated, and at the moment, no database entry can be found.

LC–MS/MS method development

For the establishment of a profiling method, the MS/MS SRM mode was employed, which has a very high specificity and sensitivity for the respective compound. Its chromatogram represents only product ions of a particular *m/z* ratio and is generated by fragmentation of a selected precursor ion. In this method, the direct infusion of synthetic peptides into the mass spectrometer was used to optimize the parameters. With the combination of them with an LC gradient program, a multi-analyte LC–MS/MS method for determination of up to eight peptides from hazelnut was developed. For each of them, the most intense SRM transition served as quantifier (Q), whereas the second and third SRM transitions were chosen as qualifiers (q_1 and q_2) for the identification of the target compound (Table 1).

Using the synthetic standard peptides, appropriate calibration curves could be created with this method. Depending on the peptide, the lowest determined concentrations were either 3.1 or 4.2 ng/mL, and the linear range extends up to 3,125 or 4,167 ng/mL, respectively. The coefficients of determination of calibration curves were between 0.9914 and 1.000.

Occurrence of selected hazelnut marker in other nut extracts

In general, the protein databases are often incomplete; therefore, all of the nuts listed as allergen in the EC-Directive 2007/86/EC (including almond, hazelnut, walnut, cashew, pecan, Brazil nut, pistachio, macadamia and peanut) were used in this study. Nuts were extracted, digested and measured with the MS method mentioned above to observe if the selected hazelnut peptides could also be found in the other nuts (Table 2). The first criteria for detection of a peptide were the mass, the retention time and three SRM transitions, which should be matched to the standards (three examples shown in Fig. 2). If there were mismatches with the theoretical results, the ratios of the SRM intensities were compared with the synthetic standards. If necessary, nut extracts were also measured in EPI mode.

According to BLAST results, theoretically, only WLQLSAER should be detected in walnut, pecan and pistachio. In the first instance, it seemed that the MS data showed different results. WLQLSAER was found in walnut

Table 2 Concentrations (microgrammes per gramme) of selected hazelnut peptides detected in digested hazelnut, walnut, pecan, pistachio and cashew samples

	Hazelnut	Walnut	Pecan	Pistachio	Cashew
GIAGLNPNLAAGLPGK	34.1	–	–	–	–
ALPDDVLANAFQISR	200	–	–	–	–
QGQVLTIPQNFAVAK	250	–	–	–	–
INTVNSNTLPVLR	397	–	–	–	–
WLQLSAER	114	6.4	–	13.7	64.2[a]
AFSWEVLEAALK	7.1	3.0[a]	21.6[a]	–	–
LLSGIENFR	5.4	–	–	–	–
ELAFNLPSR	4.4	–	–	–	–

None of these peptides could be found in digested peanut, Brazil nut, macadamia and almond samples

[a] Due to sequence homology, hazelnut peptides are falsely detected at the given concentrations

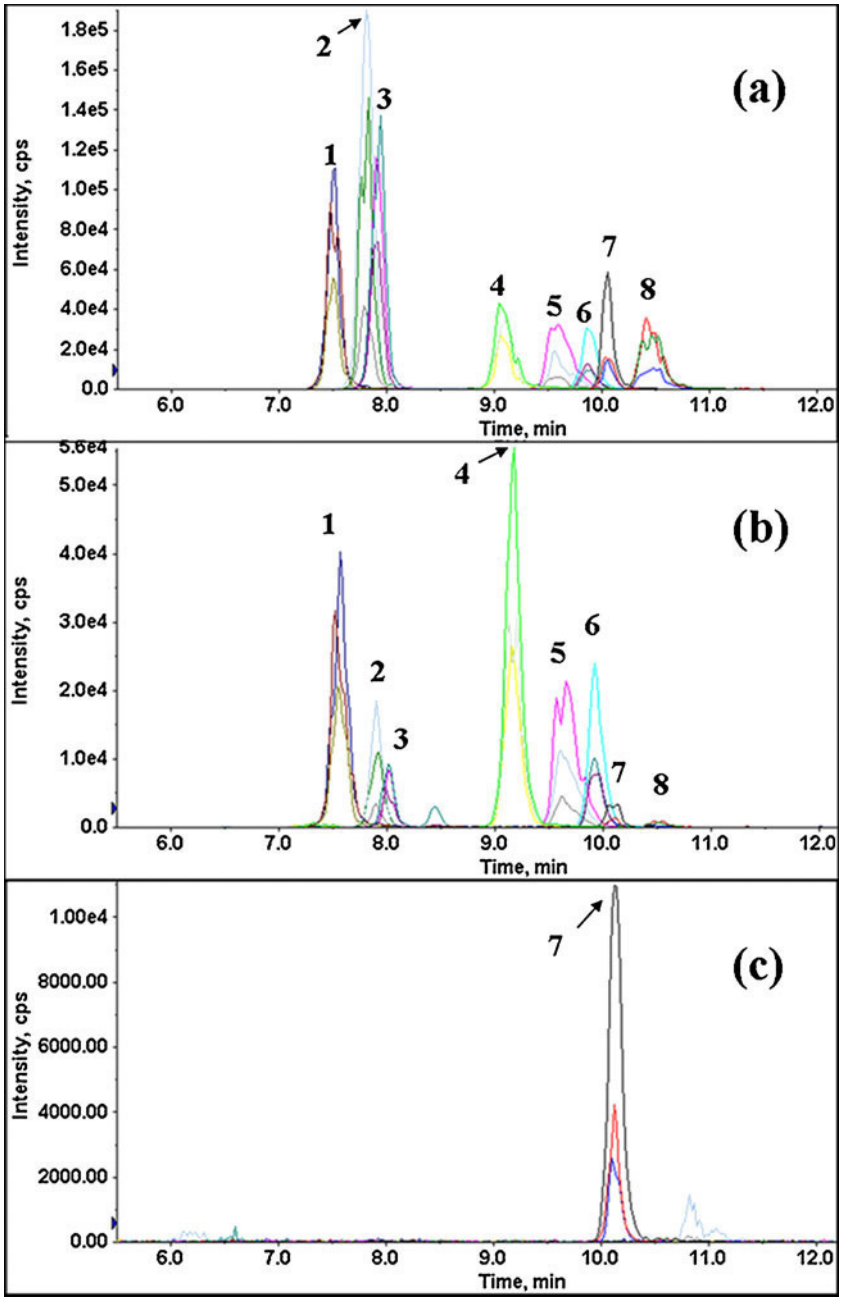

Fig. 2 Total ion chromatogram of (**a**) synthesized standard mixture, (**b**) digested HN extract and (**c**) digested pecan extract. *Peak 1*: WLQLSAER, *peak 2*: LLSGIENFR, *peak 3*: ELAFNLPSR, *peak 4*: INTVNSNTLPVLR, *peak 5*: ALPDDVLANAFQISR, *peak 6*: QGQVLTIPQNFAVAK, *peak 7*: AFSWEVLEAALK and *peak 8*: GIAGLNPNLAAGLPGK. Different transitions were shown with *different colours*

and pistachio (not in pecan) but additionally also in cashew. In contrast to BLAST information, AFSWEVLEAALK was likely detected in walnut and pecan in very low concentrations (Table 2). In this case, all of the three transitions were detected in the extracts at retention times very near to that from synthesized standard. Since these results did not fit the BLAST search results, the intensity ratios of three SRM transitions were also checked, which actually did not match with the synthetic standard.

Thus, two databases (NCBI [20] and UniProt [18]) were searched for proteins of walnut, cashew and pecan. Each found protein was in silico digested with trypsin using PeptidMass [17] and the obtained peptides were compared with our standard peptides. In cashew allergen Ana o 2, which belongs to the 11S seed storage globulin family, a similar peptide could be found: WLQLS**V**VE**K** with $[M+H]^+$ 1,002.2 Da. Therefore, digested cashew extract was measured in EPI mode (Q1, 501.9; CE, 35 V), and its spectrum was compared with the spectrum of the synthesized standard measured at the same EPI conditions, and also with the product ions obtained by in silico fragmentation using PFIA II (Fig. 3). As expected, the spectrums are very similar; however, two characteristic fragments (175.1 and 304.2) could not be found in cashew. Since all

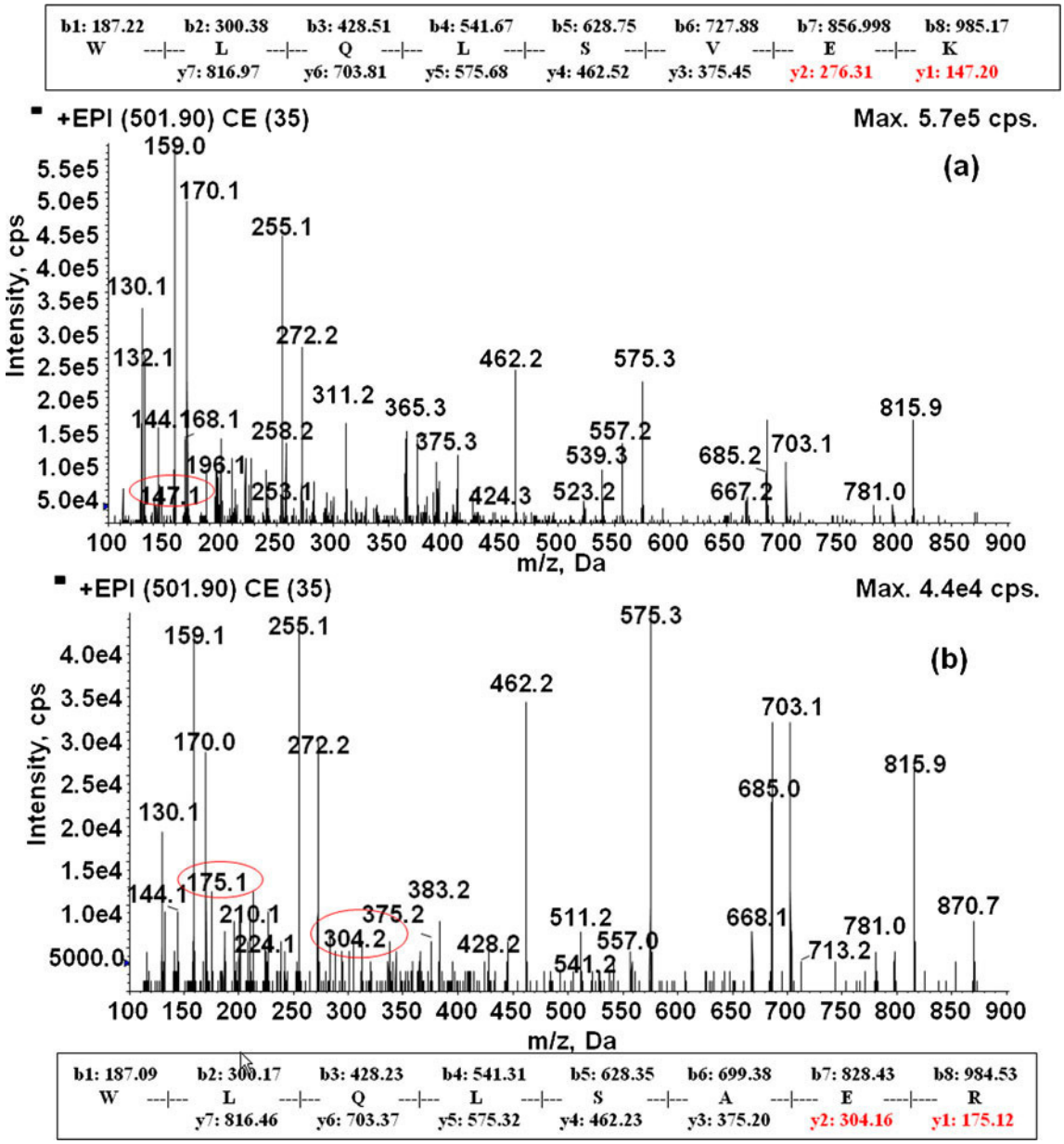

Fig. 3 Product ion spectrum ($[M+2H]^{2+}$, 501.90) of (**a**) digested cashew extract and (**b**) synthesized standard mixture. The product ions obtained from PFIA-II were shown in *boxes above or below related spectrums*. y1 and y2 are characteristic for each peptide

of the chosen transitions for WLQLSAER with MW 1,001.5 (Q1, 501.9; Q3, 159.2, 272.3 and 575.5) were from the homologue part of the WLQLSVE**K** sequence with 1,002.2 Da (a1, a2 and y5 according to PFIA-II), it could also be detected with the developed LC–MS/MS method.

The product ion spectrums of digested walnut and pecan extracts ($[M+2H]^{2+}$ *m/z*, 682.7; CE, 35 V) were also similar to the spectrum of the standard; however, the search in the database for a similar peptide to AFSWEVLEAALK in walnut and pecan proteins was not successful. It only can be speculated that in these two nuts, there is again a sequence akin to AFSWEVLEAALK, which is under investigation at the moment.

Conclusion

Eight tryptic peptides of three hazelnut allergens Cor a 8, Cor a 9 and Cor a 11, which gave high MS signals, were selected and synthesized. Using them as standards, an LC–MS/MS method in SRM mode was developed for the detection of hazelnut in food products. According to a BLAST search, only one of the four Cor a 9 peptides occurs in different nuts (walnut, pecan and pistachio). However, the MS measurements showed that this peptide occurs also in cashew. Additionally, one peptide from Cor a 11, which according to BLAST is hazelnut specific, showed a significant SRM signal for walnut and pecan as well. Using

product ion spectra, we demonstrated that the detected cashew, walnut and pecan peptides are very similar to our synthetic peptides, but not the same.

In this work, we could show that anti-hazelnut antibodies are cross-reactive also to other nuts. A reason for it is the sequence homology of proteins from different nuts, which can also be a problem for LC–MS/MS measurements for the detection of hazelnut in different food samples. However, the specificity of MS/MS full scans allows the unambiguous determination of peptides, strongly minimising the cross-reactivity encountered with immunoassays. Thus, we could develop a sensitive and selective method for the detection of hazelnut proteins in food samples, alternative to immunoanalytical methods. In further studies, we intend to use this method for the determination of trace amounts of allergic nut proteins in processed food.

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4 Conclusion

4.1 Extraction, characterisation and purification of the allergens

A major focus of allergenic food protein analysis is the detection and characterization of food allergens listed in Annex IIIa of the EC-Directive 2007/86/EC. The demand for rapid test kits, which can check the presence of food allergens in less than 60 min and in an easy-to-use manner, increased in the last years. Therefore in a review paper, the fast immunoassays and their application to food allergen analysis are discussed. Furthermore, the commercial available test kits are summarised in a table [1]. One of the most used systems are ELISA test, which have been developed for different food allergens with testing time of ca 30 min. Blocking, which affect the sensitivity and specificity of the ELISA, is one of the most important steps in developing the kits. The most effective and common used blocking solutions are proteinaceous BSA, casein and fish gelatine; however, in food allergy research, it is preferred to avoid them. As part of our work, it has been proven that polyvinylalcohol and Ficoll showed enough blocking efficiency and inhibit the binding of non-specific proteins [2]. Therefore, Ficoll has been used as common blocking buffer for further ELISAs.

Extraction and purification of allergenic proteins from foodstuffs are crucial tasks that affect the quantification of obtained results from analytical methods. Therefore in this work, different strategies and innovative extraction techniques were tried, for example using cooled acetone to defat the nuts for efficient extraction of target allergenic proteins. In case of non-commercially available proteins, such as hazelnut proteins, the purification and characterisation of allergenic proteins is of particular importance. Since one third of all anaphylactic reactions are caused by tree nut ingestion, a major focus of this work was put on the detection and characterization of nuts listed in Annex IIIa of the EC-Directive 2007/86/EC, especially hazelnut. After grinding and defatting the nuts, different extraction buffers were tried and finally ammoniac hydrogen carbonate buffer was chosen as the most suitable one. The extracts were dialysed to remove excessive salts, which disturb further gel electrophoresis that was performed for a fast control of the efficiency of extraction. The extracts were subjected to SDS-PAGE, where their proteins were separated due to their molecular weight. This allowed a profiling of extracted proteins, and the differences between the

extractions became visible. Furthermore the immunological studies such as western blot have been performed for identification of proteins recognized by antibodies.

For isolation and purification of food proteins from extracts, different chromatographic methods such as size exclusion chromatography (SEC), ion exchange chromatography (IEC), affinity chromatography, immunoaffinity chromatography and reversed phase chromatography (RPC) were used; and to obtain the best results, the combination of these methods was tested also. Thus purification protocols were established and the obtained preparations were used for the characterisation of in-house produced antibodies.

In case of hazelnut, a two-step FPLC system, affinity chromatography (Con A Sepharose) and reversed phase chromatography (ProRPC HR 5/10), delivered the most purified preparations. With a time-of-flight mass spectrometry (HPLC-ESI-TOF/MS) the molecular weight of these purified fractions was determined. These preparations were used for the characterisation of six in-house produced monoclonal and polyclonal anti hazelnut antibodies. Three monoclonal antibodies showed an identical protein recognition pattern that is analogue to the pattern of polyclonal antibody from mouse, whose spleen had been used for the production of mABs; their IgG showed stronger binding to larger proteins. Unfortunately the obtained amount of the preparations was only sufficient for some western-blot experiments and characterisation by HPLC-ESI-TOF/MS. This situation exactly demonstrates that although it is always asked for characterised standards, the needed time and effort for such natural preparations is nearly unaffordable.

During this work, also the extracts of different nuts, listed in EC-Directive 2007/86/EC as allergens, were used to check the cross-reactivity of in-house produced anti hazelnut antibodies by western blot. They cross-reacted with all extracted nuts; however, the reaction profile was not identical. The cross-reactivity of the monoclonal antibody was more restricted to the molecular weight range where the 7S storage proteins can be found. The polyclonal antibody showed cross-reactivity to proteins in the low molecular weight range, where also pollen allergens are located. Since almost all food allergens of nuts belong to the seed storage protein family and appear within few botanical families of rosids, they have homologue structure and it is difficult to prevent this cross-reactivity between the different nuts [4].

4.2 Development of mass spectrometric methods

Currently, advanced analytical methods based on liquid chromatography/tandem mass spectrometry (LC-MS/MS) are highly requested for identification and quantification of allergenic proteins. The emphasis of this work was put on developing MS-based methods for the detection of milk and hazelnut allergens in food samples. For this purpose different bioinformatic tools such as Basic Local Alignment Search Tool (BLAST), PeptideMass and Peptide Fragment Ion Analyser (PFIA) and databases like Universal Protein Resource (UniProt) and National Center for Biotechnology Information (NCBI) had to be used to get more information and to facilitate the method-development and peptide searching. Since the used LC-MS/MS is unable to characterise the molecules bigger than 3 kDa and the molecular weight of allergens are between 3-160 kDa, the enzymatic digestion had to be performed to produce peptides that can be scanned with the QTrap 4000 LC-MS/MS. Therefore characteristic peptides were sought, which can function as marker for the detection of allergens in food samples.

For method development, if the allergens are commercial available (for example milk allergens: α- and β-casein and whey proteins α-lactalbumin and β-lactoglobulin), they were bought, digested with trypsin and scanned with MS. In-silico tryptic digestion has provided preliminary information about the expected peptides. The data obtained from MS/MS spectra were evaluated and among the found peptides of these allergens, those with the highest MS-signal were chosen, synthesized and searched as marker in the food samples. Also BLAST search were performed to confirm the specificity of the peptides. The food samples underwent the same process (extraction, digestion, and measured with the developed MS-method). The synthesized peptides can be used as standard for the quantification of the peptides, found in the food samples. In case of nonexistent commercial proteins, the extracts of the allergenic food commodities (e.g. hazelnut) have been digested.

During this dissertation, two sensitive and selective methods for the detection of milk and hazelnut allergens in food samples by LC-ESI-MS/MS in SRM mode were developed. It was the first step to establish alternative and comparative methods to immunoassays for the detection of allergenic food proteins in different matrices. The HPLC separation prior to MS is relative rapid as the run lasts only 20 minutes for milk and 23 minutes for hazelnut. The LC-MS/MS in SRM mode is a very selective method with good quantification properties. Although the sample preparation is more complex

compared to immunoassays, it could be shown by spiking experiments that the extraction and digestion procedures do not have a negative influence on the results. Therefore, the used LC-MS/MS method could be a possible candidate in the process of developing reference methods for food allergen detection.

For the detection of milk, seven peptides (chain length: 5-12 amino acids) derived from tryptic digestion of four allergenic milk proteins (α- and β-casein, ALA and BLG) were chosen. According to BLAST search, only one of them (LIVTQTMK from BLG, *m/z* 467.6, charge 2+) is specific for the bovine milk; two other peptides, FFVAPFPEVFGK from α-casein (*m/z* 693.3, charge 2+) and GPFPIIV from β-casein (*m/z* 742.5, charge 1+), could be used as marker for the detection of milk from cow and water buffalo; the others markers are suitable for the detection of four major ruminate milk allergens in general. The LC-MS/MS multi-analyte method, which was developed for the simultaneous determination of these seven peptides, used them as standard for the quantification of detected peptides in the food samples. The method is able to detect the selected peptides in calibration analysis with the LODs between 1 and 11 ng/mL, depending on peptide (signal-to-noise ratio=3). The method showed low detection limit; the peptides could be measured with concentrations down to 1.1 ng/mL (50 ng/g of milk sample). Since the peptides could be found with these low concentrations in cheese and yogurt varieties, and also in chocolates, it seems that neither fermentation nor with polyphenols masked proteins disturb the detection. It has to be mentioned that in immunoanalytical methods, special extraction efforts are necessary for chocolate samples including polyphenol to avoid the protein loss. The spiking results, which were divided into standard addition to test the matrix effect and recovery calculations, were satisfying because the target peptides could be detected in the samples with the recoveries over 50%. However, it has to be mentioned here that for further trace amount determination the method has to undergo a complex validation procedure [3].

For the development of hazelnut detection method, at first the hazelnut was extracted, digested and measured with MS/MS by direct infusion. Eight tryptic peptides from three major hazelnut allergens Cor a 8, Cor a 9 and Cor a 11 , which showed the highest MS-signal, were selected and used as marker for the development of a LC-MS/MS method in SRM mode. These peptides were synthesised and used for the calibration curves, where the lowest determined concentrations were either 3.1 or 4.2 ng/mL (depending on peptide) and the linear range extends up to 4167 ng/mL. The

determination coefficient of calibration curves were between 0.9914 and 1.000. According to BLAST search, only one of these peptides (a Cor a 9-peptide) occurs in different nuts (walnut, pecan and pistachio). To confirm these results, eight other nuts listed in Directive 2003/89/EC were extracted, digested and measured with the developed MS method. The MS measurements showed that the Cor a 9-peptide occurs also in cashew. Additionally, one peptide from Cor a 11, which according to BLAST is hazelnut specific, showed a significant SRM signal for walnut and pecan, as well. However with using the product ion spectra, it could be demonstrated that the detected cashew, walnut and pecan peptides are even though very similar to our synthetic peptides, but not the same. Since almost all food allergens derived from nuts belong to the seed storage protein family, they have similar sequence and homologue structure, which can be a problem for both immunobased and LC-MS/MS methods developed for the detection of hazelnut in different food samples. However, the specificity of MS/MS full scans allows the unambiguous determination of peptides and minimises the cross-reactivity compared to immunoassays. Comparing these results, it could be shown that with this sensitive and selective LC-MS/MS method in SRM mode the specific detection of hazelnut is possible as alternative to the common immunoassays. The next step will be using this method for the determination of trace amounts of hazelnut allergens in processed food samples, which lead to establishing a reference method [4].

Concluding remark

Nowadays production of food became more and more important and consumers want to take accurate information about the origin and ingredient of food products and demand more rigorous food safety testing. Food safety is a result of combination of several activities along the food chain from farmer to manufacturer and consumer to minimise the contaminants in final food products. In this context, establishment of methods for their identification and characterisation is essential. However in case of food allergen detection, the cooperation of different scientific field such as food technology, biochemistry, immunology, analytical chemistry, allergology, and food chemistry is important to develop and optimise different analytical methods. Depending on the proposed aim, both rapid immunoassay and costly MS-based methods are and will be necessary.

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